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VOL. 10 æ 1958 æ NO. 2

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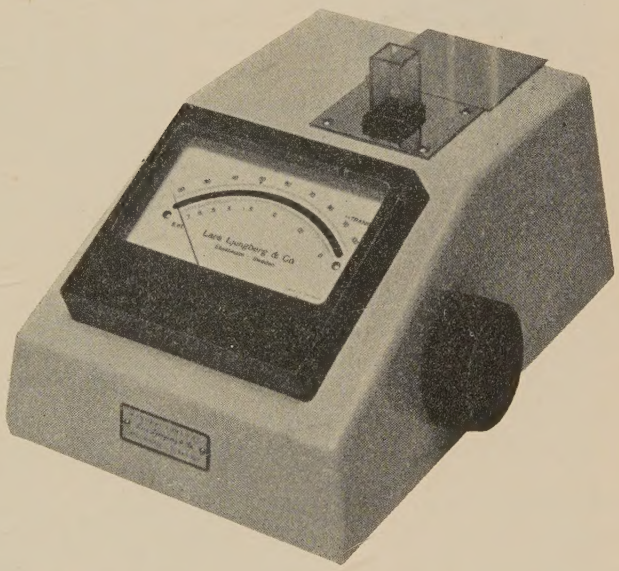

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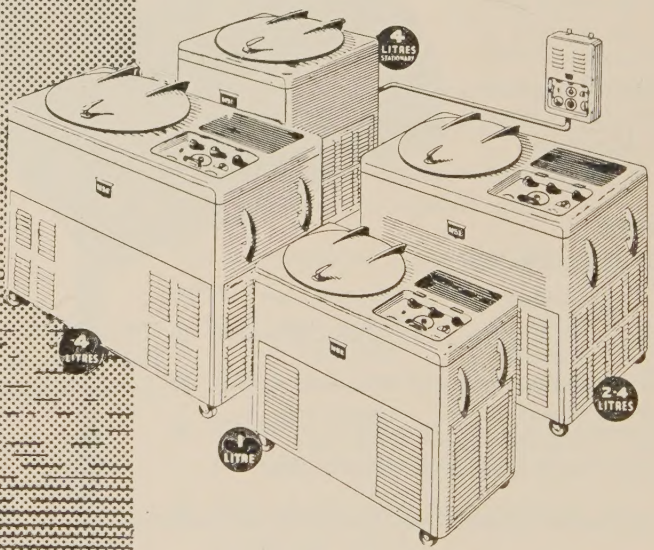

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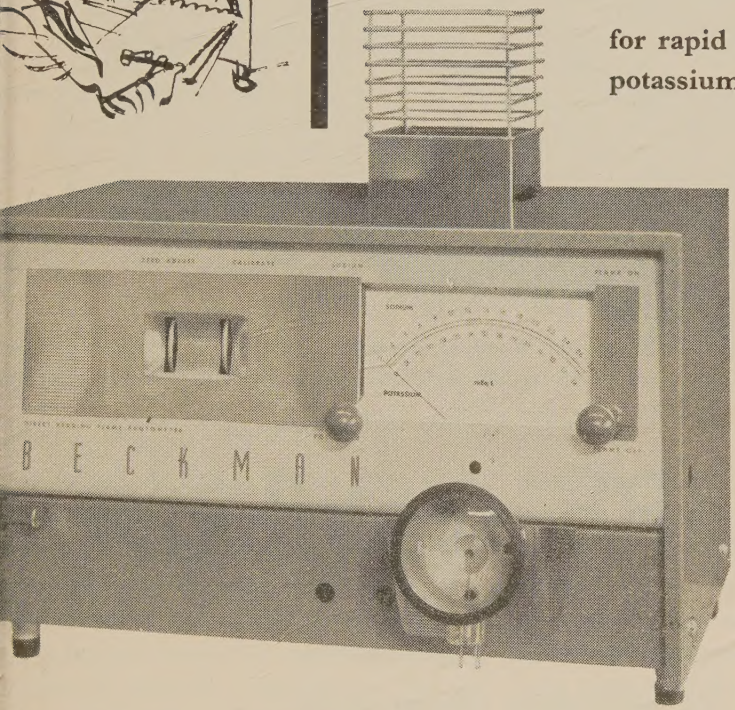

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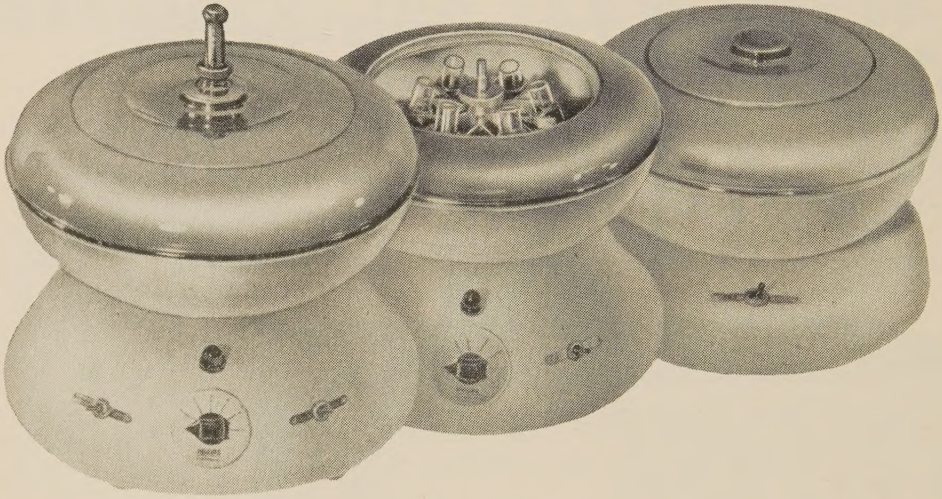

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Vol. 10 · 1958 · No. 2

ndinav. J. Clin. & Lab. Investigation 119—121, 10, 1958.

PLASMAPHERESIS IN THE PREPARATION OF HYPERIMMUNE SERUM

By R. LUNDSTRÖM and O. RAMGREN

rom the Pediatric Clinic of Karolinska Institutet at Kronprinsessan Lovisas Barnsjukhus, The State acteriological Laboratory, and the City of Stockholm Blood Transfusion Centre, Stockholm, Sweden

(Received for publication November 4, 1957)

Plasmapheresis means that, after withwing blood, the corpuscles are separated m the plasma and reinjected into the nor. The advantage of this procedure is t considerably larger quantities of plasma n be obtained from the same donor, since interval between withdrawals can be rtened. Plasmapheresis has been used retedly in animal experiments. The method s applied for preparation of human plasma Grifols-Lucas (1952), who carried out smapheresis on the same donors once a ek for a year, without any demonstrable urious effects. This is a matter of great portance in the preparation of so-called perimmune gamma globulin, which is obned through fractionation of convalescent od, or of blood from donors hyperimnized by repeated doses of vaccine.

When collecting serum or plasma for imnization purposes, it has generally been ssible to recover a maximum 250 ml per month from the individual donor. With respect to convalescent serum, it has been the rule to withdraw blood not more than four times from the same patient in the course of between three weeks to four months after recovery. Consequently, in these cases, we have been able to obtain only a total 800 to 1,000 ml of serum or plasma, respectively, from one patient.

With a view to investigating the possibility of increasing the supply of convalescent serum, tests with plasmapheresis were started in June 1956 in a few donors with a recent history of rubella. After withdrawing about 500 ml of blood into a glass vessel containing ACD solution, the blood was centrifuged immediately, the plasma kept, and the remaining suspension of corpuscles reinjected into the donor.

The demand for pertussis hyperimmune gamma globulin has risen appreciably in Sweden during the past few years. We there-

fore considered it motivated to investigate whether larger quantities of plasma could be collected from voluntary donors hyperimmunized with pertussis vaccine.

METHODS

Ten donors, whose serum after hyperimmunization with pertussis vaccine SBL* agglutinated *Haemophilus pertussis* in a titre of 1 : 1,600 to 1 : 25,600, volunteered for the experiments.

At the beginning of the experiment, 1 ml of alum-precipitated pertussis vaccine (SBL No. 285), corresponding to 20 billion dead bacteria, was injected subcutaneously. After a week, about 500 ml of blood were withdrawn from each donor into bottles containing ACD (USP sol 1) solution. The bottles were stored at a temperature of $+4.5 \pm 0.5°$ C. One week later, the plasma was aspirated under sterile conditions. Immediately afterwards, a further 500 ml of blood were withdrawn from the same donors. The corpuscles from the blood withdrawn one week earlier were then reinjected through the same cannula inserted in a cubital vein used for the removal of blood.

Five of the 10 donors underwent plasmapheresis once a week for 10 consecutive weeks, and five donors once a week 8 times in succession. Plasmapheresis was thus performed altogether 90 times. An additional 1 ml of pertussis vaccine was given every four weeks.

RESULTS

The *agglutination titre* to *H. pertussis* ranged from 1 : 800 to 1 : 25,600 in the 90 plasma portions. In 85 portions it was 1 : 1,600, and in 5 portions 1 : 800. The latter values refer in all cases to plasma portions obtained four weeks after immunization.

The *hemoglobin concentration* of the blood showed a somewhat prolonged course of recovery. The mean value, which was 13.8 g/100 ml at the beginning of the experiment, fell after one week to 11.0 g/100 ml. After five weeks, the mean value was 12.2 g/100 ml (2 donors having over 14.4 g, 3 between 13.5 and 14.4 g, and 5 between 11.0 and 12.2 g/100 ml). Both the individual values and the mean value showed a rising tendency with each successive week. The *serum iron concentration* was determined in each donor after four weeks' plasmapheresis, and showed decreased values, the mean value being 42 γ/100 ml. One month after the end of the experimental period, the concentration had regained the normal level. Electrophoretic determination of the *serum proteins* was performed at the end of the experimental period, and showed entirely normal values.

During the whole experimental period, all the donors carried out their normal work without complaining of any impairment in health.

DISCUSSION

Using plasmapheresis with the method described during a period of 8 to 10 weeks, plasma was obtained with a satisfactory concentration of antibodies agglutinating *H. pertussis*. The state of health of the donors did not seem to be impaired. With the technique applied earlier, *i. e.*, withdrawal of blood at most once a month, the quantity of plasma would have been only one-fourth of that recovered by the present method. Since a relatively large decrease in the serum iron concentration was observed in a few cases, and the return of the hemoglobin concentration to a normal level was delayed in some, a careful check should be kept on these values in subsequent trials.

The serum proteins, determined by paper electrophoresis, showed a normal picture.

* State Bacteriological Laboratory.

Recently, Smolens, McGee & Hunter 956) published a description of an appa- us for convenient plasmapheresis in hu- n donors. They stated that the technique ild also be applied for collection of hyper- mune plasma. Their apparatus is, however, pensive and, for this reason, among others, not at present be used in ordinary hospital od services. The technique described in s paper can be used without any special uipment.

The interval at which plasmapheresis is rformed can be varied from one to three eks. The method permits maximum utili- ion of hyperimmune serum from convales- nt patients as well, without any detrimental fect on their state of health.

SUMMARY

For preparation of pertussis hyperimmune mma globulin, 10 donors underwent plasmapheresis once a week for 8 to 10 consecutive weeks. Plasmapheresis was performed altogether 90 times. In 85 of the 90 plasma portions, the agglutination titre to *H. pertussis* was satisfactory ($\geqq 1:1{,}600$). Apart from a somewhat prolonged course of recovery in the hemoglobin count, no untoward effects were demonstrable. The individual values and proportions of the plasma proteins, determined by paper electrophoresis, were normal.

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Scandinav. J. Clin. & Lab. Investigation 122—132, 10, 1958.

THE USE OF THE ISOTOPE Cr^{51} FOR STUDYING DESTRUCTION OF RED CELLS IN HEALTHY HUMANS

By S. BIRKELAND

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(Received for publication November 13, 1957)

Under physiological conditions red cells are thought to be released from the bone marrow (production) at an average daily rate, to remain in the circulation (survival) for 100—130 days and then to disappear (destruction) (Ashby, 1948; James III, Abbot, Norberg, Birkeland & Evans, 1953; Eadie & Brown, 1953; Crosby, 1954; Mollison, 1956).

The Ashby technic (Ashby, 1919) in its various modifications is generally accepted as a reference method for determination of survival time. However, the method can not be used for studying the destruction of an individual's red cells in his own organism, and difficulties may arise from antibody formation (Mollison & Cutbush, 1955).

Gray & Sterling (1950) introduced the method of labelling the individual's own erythrocytes with Cr^{51} and also used the tagged cells for determination of total red cell mass (Sterling & Gray, 1950). Ebaugh, Emerson & Ross (1953) used Cr^{51}-tagged red cells for studying cell survival in comparison with the Ashby technic. Since then the Cr^{51}-method has been dealt with in several reports (referred to in the discussion). The usefulness of the latter method is dependent upon whether the tagging procedure itself causes any change in cell survival, and whether the rate of Cr^{51}-elution from intact erythrocytes takes place at a uniform rate after infusion. For describing red cell turnover in many clinical conditions it is of great importance to follow cell destruction in the host organism.

Previously reported data were mainly derived from Cr^{51}-concentration in whole blood or red cells. The purpose of the present study was to determine the Cr^{51}-disappearance curve by following the total amount of Cr^{51} in the circulation, employing repeated determinations of total red cell volume and correction for samples removed. Based on such curves, obtained in healthy individuals, the Cr^{51}-elution rate (release from intact cells) was evaluated, numerically expressed and corrected for in order to evaluate the red cell life span.

MATERIAL AND METHODS

Data were obtained in 10 healthy volunteers, 8 males and 2 females, 23—57 years of age. None had received a blood transfusion before. Physical and urine examination as well as blood values revealed normal findings.

* This study was conducted under U.S. Public Health Grant H-1142 as a Research Fellow in Surgery at the Medical College of Virginia, 1952—1955. The preparation of the data was supported by a grant from Nordisk Insulinfond, Gentofte, Denmark.

Hematocrit (Hct) expressed as per cent, hemo-lin (Hgb) as grams/100 ml blood and mean cor-cular hemoglobin concentration (MCHC) were rmined as previously described (Birkeland, 8, b). Total red cell volume (RCV) was deter-ed by the T-1824-technic (Birkeland, 1958, b).

Cr^{51}-tagging of red cells

0—300 ml blood was drawn by gravity (No. 18 lle) into ACD-solution (Abbott Laboratories, th Chicago, Ill.) in proportion ACD-solution/ od = 1/4 in non-siliconized rubber-capped bottles. otal of 60—100 μc Cr^{51} was added as sodium omate (Abbott Laboratories, Rachromate) to-ing 10 μg or less of chromium per ml of red s.

The blood was mixed by gentle tilting of the le at intervals for 40—60 minutes at room tem-ture (range 22—36° C), then placed in a cold m (+4° C) for 12—20 hours. During this period cells had sedimented, and now the supernatant sma and white cells were removed by suction. d saline (+ 4° C) with approximately 75 mg of orbic acid* (Read, 1954) was added to about 3 times the volume of red cells. Red-cell-saline xture was spun at 3000 rpm for 30 minutes in cold-room. Supernatant saline and remaining ite cells were sucked off, and after resuspension old saline (to a volume approximately 2—3 times volume of cells) the Cr^{51}-red cell suspension s ready for infusion without any previous warm-up.

Infusion and sampling

A measured volume of well-mixed Cr^{51}-tagged cell suspension was infused by gravity, or very wly from a calibrated 50 ml syringe (No. 18 edle). 10—30 minutes after the end of infusion Cr^{51}-tagged cells, 5 or 10 ml T-1824 solution proximately 0.15 per cent) was injected through same needle from a calibrated syringe. 3—6 ious samples (8—11 ml each) were then drawn hout stasis at intervals during the next 30—50 nutes into test-tubes containing Heparin (0.5 mg/ blood). — Repeated determinations of red cell ume (T-1824 technic) and Cr^{51}-activity in red ls were made during the following 45—80 days.

* Sharp and Dohme for intravenous use.

Counting

From each sample 5 ml of whole blood was pipetted at once into a standard test tube, hemolyzed with saponine and kept in the ice-box until the time of counting when the tube was placed in a well scintillation counter (Nuclear). Aliquots of 5 ml from suitable dilutions of the infused red cell suspension and usually of its supernatant and of the serum (obtained during first few days after infusion) were counted together with the blood samples, usually at the end of the period of observation.

A rubber-stoppered and sealed Cr^{51}-standard was counted along with the samples, which by reference to this standard were corrected for decay and change in counter sensitivity. Results were expressed as counts per min. per ml of red cells (c/min/ml RCV) after having subtracted the activity in serum. Background activity was about 300 counts/min. 20,000 counts were obtained for background and for each of the samples.

Calculations

Total amount of Cr^{51}-activity remaining in the circulating red cell volume (RCV) was quantitatively expressed as follows: c/min./ml RCV (C_t) at any time t, in days after transfusion, multiplied by the circulating red cell volume (RCV_t) measured at the same time ($N_t = C_t \times RCV_t$) was expressed as per cent of initial value ($N_0 = C_0 \times RCV_0$) at $t = 0$ and plotted against time. Where C_t was obtained without simultaneous determination of total red cell volume, RCV_t was estimated by interpolation between the preceding and following RCV-measurement.

Initial value for C_0 was the average of values obtained during 60 minutes after infusion. If there was any indication of a slope the last values (30—60 minutes) were used.

RESULTS

In one experiment 800 ml blood was drawn from a donor (B.W.) and tagged with Cr^{51} as described. When the resulting tagged red cell suspension was ready for infusion it was divided in four equal portions. The donor

Table I. *Data from 10 healthy controls.*

a	b		c	d	e	f	g
Initials, age and sex	Per cent Cr^{51}-survival ($N_0 = 100$ per cent)		50 per cent survival on day ($N_0 = 100$ per cent)	Red cell life span from corr. Cr^{51}-survival ($N_1 = 100$ per cent)	Half time Cr^{51}-elution days ($N_1 = 100$ per cent)	Ratio A	Chromium μg pr. ml red cells
	Day 1	Day 2					
B.W., 34 M	96.1	97.9	29.0	123	54.5	1.10	3
A. K., 30 M	94.5	94.2	28.0	116	47.0	1.12	3
B. C., 27 M	98.0	95.8	28.0	99	44.0	0.93	3
A. G., 28 M	95.5	94.5	28.5	125	54.0	1.15	3
H.H., 44 M	95.0	90.4	25.5	127	46.0	1.00	4
G. D., 55 F	94.4	—	34.0	112	55.0	1.07	2
C. M., 23 F	95.3	—	28.0	118	56.0	1.02	4
E. C., 48 M	97.4	95.6	27.5	105	45.5	0.93	10
W. S., 57 M	95.7	95.7	26.0	105	48.0	1.02	6
O. S., 53 M	—	93.2	29.5	115	50.5	1.04	4
Mean	95.8	94.7	28.4	114.5	50.1	1.04	
S. D.	1.2	2.2	2.3	9.3	4.5	0.08	

himself (B.W.) received one aliquot, and of the remaining three one portion was given to each of three compatible recipients (A.K., B.C. and A.G.). All were of group O — Rh_1 — Kell neg. — MN. Direct cross-match at room temperature and at 37° C as well as indirect Coombs test showed compatibility in all respects.*

In 6 other healthy control individuals autotransfusion of tagged cells was given.

Results from the first group (donor and three recipients) fell within the range of the latter group (6 autotransfusions), and therefore data were pooled.

When the initial value is taken as 100 per cent ($N_o = 100$ per cent), the percentage of Cr^{51} remaining in the circulating red cell volume on days 1 and 2 after infusion is shown for the 10 controls in column b, Table I, and in column c the time is given before 50 per cent value was reached. The resulting slope of all values is shown in Fig. 1.

It was assumed that by aging *per se* the survival curve of the tagged cells would show a linear slope and reach zero value at about 100—130 days, *i. e.* the range of the life span for normal human erythrocytes. Furthermore, the handling of the cells and the amount of chromate added were assumed not to interfere with their survival (See Discussion).

If the observed values (N_t) are corrected for the linear aging expressed by $N_o\left(1-\frac{t}{T}\right)$, the remaining curve will be dscribed by

$$\text{(I)} \qquad f(t) = \frac{N_t}{1 - \frac{t}{T}}$$

where $N_o = 100$ per cent = Cr^{51}-activity present in circulating red cell volume at time zero, and N_t is the corresponding value at time t expressed as per cent of N_o.

* The author is very much obliged to Mrs. M. Waller, Rh-laboratory, Medical College of Virginia, Richmond, Va., for her kindness and cooperation in doing the serology testing.

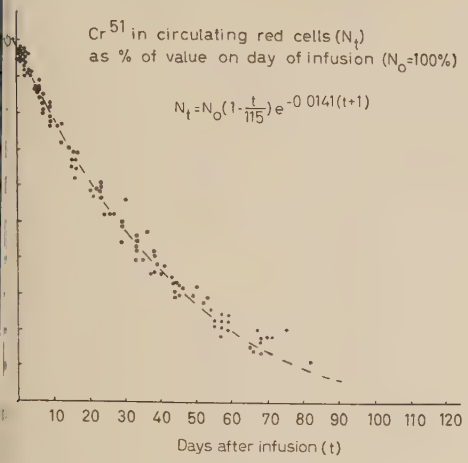
Cr 51 in circulating red cells (Nt)
as % of value on day of infusion (No=100%)
Nt=No(1-t/115)e-0.0141(t+1)
10 20 30 40 50 60 70 80 90 100 110 120
Days after infusion (t)

Fig. 1.

= days after infusion of tagged cells.
= the life span of normal red cells.
ues for T were tried over the normal
ge. For T = 115 days the values formed
easonably good linear slope on semiloga-
mic paper (Fig. 3), and the expression
became

$$\frac{N_t}{1-\frac{t}{115}} = N_o \times e^{-0.0141(t+1)}$$

h a half-time of 49 days, range 45 to 54
s (analyzed graphically). The resulting
ation for the description of the observed
appearance of Cr^{51}-tagged red cells ("ob-
ved Cr^{51}-survival") thus became (Fig. 1)

$$\text{I)} \qquad N_t = N_o\left(1-\frac{t}{115}\right)e^{-0.0141(t+1)}$$

of the observed 108 values fell within
5 per cent from this curve.
f the value on day one after infusion (for
5. value on day 2 was used as no measure-
nt was made on day one) was taken as
per cent ($N_1 = 100$ per cent) and the
ne treatment of data was applied, the dis-
earance of tagged cells was well described
(Fig. 2)

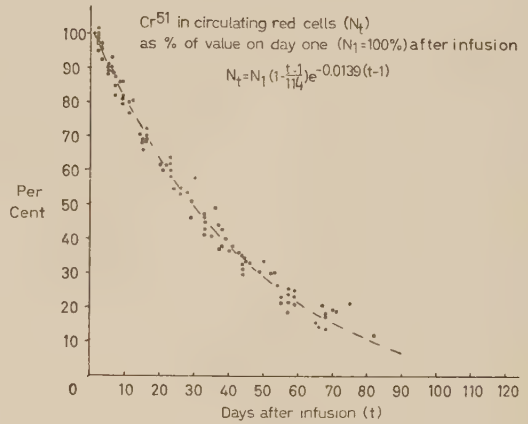
Cr51 in circulating red cells (Nt)
as % of value on day one (N1=100%) after infusion
Nt=N1(1-(t-1)/114)e-0.0139(t-1)
Per Cent
100 90 80 70 60 50 40 30 20 10 0
10 20 30 40 50 60 70 80 90 100 110 120
Days after infusion (t)

Fig. 2.

$$\text{(IV)} \qquad N_t = N_1\left(1-\frac{t-1}{114}\right)e^{-0.0139(t-1)}$$

with a half-time of 50 days (range 44—56 days) for the exponential component, Table I, column e. 94 of the observed 98 values fell within ± 5 per cent from the calculated curve. The values for

$$\text{(V)} \qquad \frac{N_t}{1-\frac{t-1}{114}} = N_1 \times e^{-0.0139(t-1)}$$

are shown in Fig. 4.

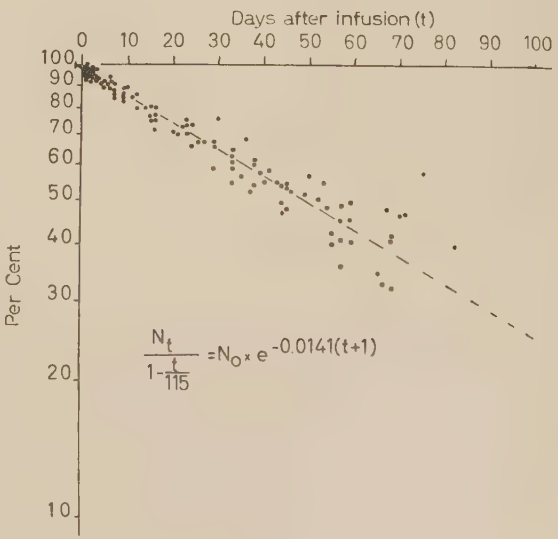

Fig. 3.

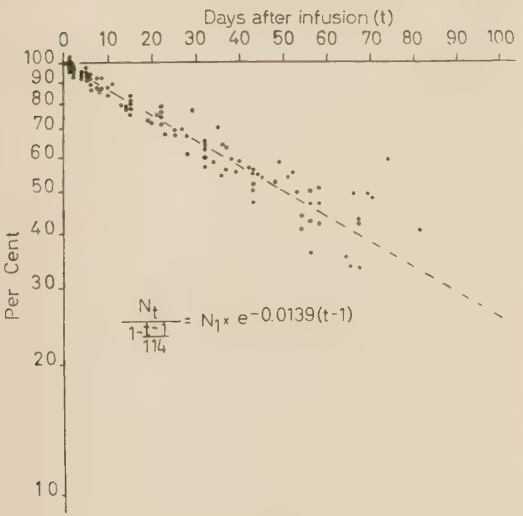

Fig. 4.

The total amount of red cells removed as samples over the first 50 days expressed as per cent of the individual's circulating red cell volume amounted to 5.2 per cent (range 3.3—6.9).

By correcting for sampling as described below, the above expressions III and IV respectively became:

$$\text{(VI)} \qquad N_t = N_o \left(1 - \frac{t}{115}\right) e^{-0.0136(t+1)}$$

with a half-time of 51 days for the exponential component, and

$$\text{(VII)} \qquad N_t = N_1 \left(1 - \frac{t-1}{114}\right) e^{-0.0133(t-1)}$$

with a corresponding half-time of 52 days.

The latter equations (VI and VII) would appear as the most correct description of the Cr^{51}-disappearance from infused tagged red cells in the present 10 healthy controls as a group. Deviations of observed values from the line $N_1 = 100$ per cent (Equ. IV) are shown as per cent in Fig. 5.

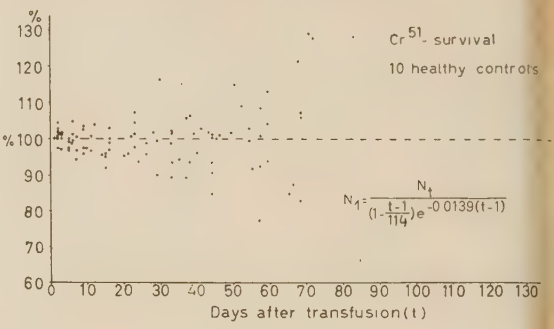

Fig. 5.

When the observed N_t-values (Fig. 2) were divided by the exponential component of Equ. IV, corrected Cr^{51}-survival was obtained corresponding to the linear aging process, as shown in Fig. 6 which corresponds to the data plotted in Fig. 2. Calculated linear regression lines for these slopes cut the abscissa at values for t given in Table I, column d.

If correction for repeated removal of larger samples is neglected, the observed Cr^{51}-survival when corrected for elution may tend to show a curvi-linear slope that falsely suggests an extrinsic hemolytic process.

The material as presented in Fig. 6 already includes correction for sampling (Equ. IV). However, if corrected Cr^{51}-survival is calculated from other material by applying the elution half-times of 51 (Equ. VI) or 52 days (Equ. VII), correction should be made for sampling before comparison is made with Fig. 6. Such correction may be made for each value of N_t as follows:

If S ml of red cells of specfic activity C are removed at time $t - t_s$, then corrected N_t-values may be obtained stepwise according to the expression:

$$\text{Corrected } N_t = N_t + S \times C \left[1 - \left(1 - \frac{N_t}{N_o}\right)\right.$$

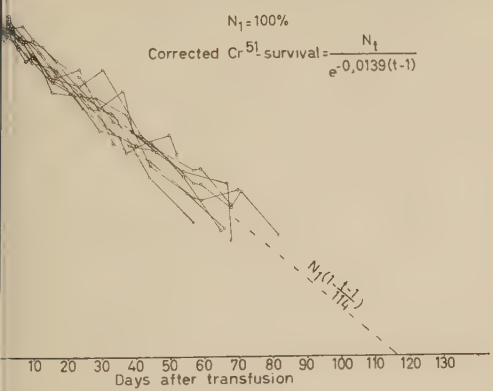
N_1=100%
Corrected Cr^{51} survival = $\frac{N_t}{e^{-0,0139(t-1)}}$
$N_1(0.\overline{t-1})$
10 20 30 40 50 60 70 80 90 100 110 120 130
Days after transfusion

Fig. 6.

DISCUSSION

Most published reports at the present time (Ebaugh *et al.*, 1953; Necheles, Weinstein & LeRoy, 1953; Read, 1954; Read, Wilson & Gardner, 1954; Weinstein, Spurling, Klein & Necheles, 1954; Mollison & Veall, 1955; Hollingsworth, 1955; Donohue, Motulsky, Hallett, Pirzio-Biroli, Viranuvatti & Finch, 1955; Ebaugh, Emerson & Rodnan, 1955; Hollie & Brown, 1955; Gibson & Scheitlin, 1955; Hughes Jones & Mollison, 1956; Burke, McAlister & Prankerd, 1956), but not all (Sutherland, McCall, Groves & Muirhead, 1954), generally agree that following the transfusion of Cr^{51}-tagged, fresh, normal cells into healthy individuals the Cr^{51}-activity is more rapidly lost from the circulation than the true rate of cell removal as measured by the differential agglutination technic. *In vivo* aging shows the same pattern (Hughes Jones *et al.*, 1956). The disappearance of Cr^{51} is assumed to be caused by linear aging cell by a not so clarified process of elution. The fundamentals of the analysis of red cell survival curves are described by others (Callender, Powell & Witts, 1945 and 1947; Ernhorst, 1951; Sheets, Janney, Hamilton & De Gowin, 1951).

The present data support the interpretation outlined above, assuming the elution process to be represented by the exponential factor in equations VI and VII. According to these expressions the amount of Cr^{51} eluted from the intact cells is a constant percentage per day (equal to 1.35 and 1.32 per cent respectively) of the Cr^{51} contained in the circulating red cell volume at any time after transfusion with the exception of the first 24 hours. During this period, the present data indicate an elution rate (or cell loss) approximately twice the ordinary rate (Fig. 1, Equ. III).

The present description (Equ. VI and VII) of Cr^{51}-disappearance in healthy controls differs only slightly from the observations of others with respect to percentage loss during the first 24—48 hours ("early loss") and the rate of Cr^{51}-elution over an extended period of observation.

The difficulties in interpreting the Cr^{51}-survival curves may arise from the fact that the basic nature of Cr^{51}-binding in and Cr^{51}-release from the red cells is unknown. The globin portion of hemoglobin (Sterling *et al.*, 1950; Necheles *et al.*, 1953) as well as washed red cell ghosts (Lionetti, Rees, Healy, Walker & Gibson, 1955) take up the tag. By paper electrophoresis and radio-autography of red cells hemolyzed by freezing and thawing, Cr^{51}-activity could not be separated from the hemoglobin (Birkeland, 1958, c).

Factors influencing the course of the Cr^{51}-survival curve for normal red cells

Effect of the $Na_2Cr^{51}O_4$ added. Metallic chromium amounting to 9.5 μg/ml of whole blood (Ebaugh *et al.*, 1953), 18 μg received per ml of red cells (Hughes Jones *et al.*,

1956) or up to 23.5 μg/ml red blood cells (Donohue *et al.*, 1955) was not observed to cause detectable changes of the Cr^{51}-survival *in vivo*.

However, 5 μg of intracellular chromium per ml of red cells was found to increase the potassium efflux above control levels during *in vitro* incubation (Gibson *et al.*, 1955). On the other hand, up to 30 μg Cr/ml of whole blood was not found to have any significant effect on mechanical fragility during *in vitro* studies while the double concentration caused increased hemolysis (Necheles *et al.*, 1953). 35 μg Chromium per ml of whole blood was found likely to change the 24 hours survival (Ebaugh *et al.*, 1953). When the cells received 35—40 μg Chromium per ml, survival was changed *in vivo* (after approximately 15 days) and in addition methemoglobin-formation was promoted (Hughes Jones *et al.*, 1956). Concentration of metallic chromium above 50 μg/ml red cells caused excessive destruction (Donohue *et al.*, 1955). No influence on fragility was noted *in vitro* from 4 μcCr^{51}/ml of blood (Necheles *et al.*, 1953). Mollison (1956) suggested that damage to red cells may be caused by the oxidizing effect of chromate.

Available evidence indicates that it is primarily the higher concentration of Chromium as a metal and not its irradiation effect that is toxic to the red cells over the range covered by the reports mentioned.

From the *in vivo* studies it would seem that cell survival would not change if a maximum of 20 μg Chromium per ml whole blood is added for incubation prior to transfusion. The *in vitro* studies by Gibson *et al.* (1955) suggest that the dose should preferably be 5 μg or lower per ml of red cells.

In the present series of measurements the added $Na_2Cr^{51}O_4$ for incubation contained 10 μg or less of Chromium per ml of red cells (Table I, column g).

Effect of in vitro handling of the red cells.

In vivo survival (differential agglutination) does not appear to be impaired by repeated *in vitro* washing of red cells with isotonic saline (Chaplin, Crawford, Cutbush & Mollison, 1954), but washing might affect the *in vitro* release of P^{32} from P^{32}-tagged red cells (Chaplin, 1954).

No increased early post-transfusion loss was found if Cr^{51}-tagged red cells were washed in isotonic saline 10—15 times, although the subsequent survival was slightly above the values obtained with Cr^{51}-cells that were washed 2—3 times (Hughes Jones *et al.*, 1956).

An unexplained early loss of Cr^{51} amounting to 10 per cent after previous washing with saline could be reduced to 5 per cent when washing was not employed (Donohue *et al.*, 1955).

Mean survival time was found (Ebaugh *et al.*, 1955) somewhat shorter (93 days) when washed cells were used as compared with unwashed cells (127 days).

It has been suggested that a definite drop in the Cr^{51}-activity within the first 24 hours after infusion was a result of damage to the blood preparatory to and during infusion (Eadie *et al.*, 1955).

Tagging of leukocytes *in vitro* is known to take place to a somewhat unpredictable extent (McCall, Sutherland, Eisentrant & Lanz, 1955; Birkeland, 1958 c). Tagging of platelets also occurs (Aas & Gardner, 1956). The disappearance of Cr^{51}-tagged red cells is

slower than for Cr^{51}-tagged leukocytes platelets. Failure to remove these components prior to infusion of red cells may e an erroneously fast disappearance dur- the first days following transfusion. This thought to be of importance especially n dealing with blood from burned pa- ts where leukocytosis was generally pre- (Birkeland, 1958 c).

he method developed by Read (1954) ts the cells very gently without washing. wever, the excess radioactivity in plasma nfused and the leukocytes and platelets not removed.

he influence of anticoagulants used parin or ACD-solution) with respect to vival does not appear to be of obvious ortance (Necheles *et al.*, 1953; Mollison *l.*, 1955).

hese reports would indicate that the *in o* handling (beyond the mere adding of $Cr^{51}O_4$) of the erythrocytes (particularly ed cells) may cause detectable changes of Cr^{51}-survival curve early after transion as well as during its subsequent rse. Therefore, gentleness and a careful ndardization of the *in vitro* procedure is icated. During the present study this was ed at as well as removal of excess Cr^{51} plasma and all visible traces of leukocytes platelets.

Other factors. It is conceivable that a her concentration of Cr^{51} may be present plasma proteins coating the tagged red s (Mollison *et al.*, 1955; Piper & Ruhenth-Bauer, 1956; Jandl & Simmons, 1957) compared with their diluted supernatant which activity was measured. Most likely h protein would be exchanged fairly ra- y *in vivo* (Slater & Sass-Kortsak, 1956).

Presence of a significant quantity of short-lived cells tagged in a normal cell population is a possibility that would explain an early loss of the Cr^{51}-tag (Hughes Jones *et al.*, 1956).

Selective tagging of different age groups of cells has not been substantiated as yet (Eadie *et al.*, 1955; Hughes Jones *et al.*, 1956; Donohue *et al.*, 1955). Finally, a delayed mixing of tagged cells with the total untagged RCV was suggested by Ebaugh *et al.* (1955).

Cr^{51} released from destroyed cells does not tag other cells *in vitro* (Ebaugh *et al.*, 1953) or *in vivo* in humans (Mollison & Cutbush, 1955) and dogs (Birkeland, 1958c).

The one hundred per cent reference point for expressing survival

Cr^{51}-activity per ml of red cells has been obtained from samples drawn 10 minutes to one hour after infusion of tagged cells (Read *et al.*, 1954; Mollison *et al.*, 1955; Hughes Jones *et al.*, 1956) or in some studies (Necheles *et al.*, 1953; Sutherland *et al.*, 1954) from samples drawn 24 hours after infusion. The 24-hours reference point may cause increase in the 50 per cent survival time (Mollison *et al.*, 1955).

In this presentation the values obtained for

$$A = \frac{C_0 \times RCV_0}{\text{Total } Cr^{51}\text{-activity in the infused red cells}}$$

are given in Table I, column f. As can be seen, the determined values are on the average actually slightly above the expected 100 per cent. It is concluded that no measurable loss of Cr^{51}-tagged cells took place during infusion.

By selecting the 100 per cent value as $N_0 = C_0 \times RCV_0$ and by using the same

method throughout for measuring RCV_t at intervals, any changes in RCV or PV will not influence the survival results, as $N_t = C_t \times RCV_t$. Serious distortion of the Cr^{51}-survival curve with greater changes in RCV is thereby obviated (Birkeland, 1958 a, c).

The course of the Cr^{51}-survival curve

The early phase (1—2 days following transfusion).

From Table I it is seen that during the first 24 hours in the present controls 4.2±1.2 per cent of Cr^{51}-activity was lost, which is more than would be expected from the aging of normal red cells. Similar findings are commented on by several authors (Mollison *et al.*, 1955; Donohue *et al.*, 1955; Ebaugh *et al.*, 1955; Eadie *et al.*, 1955; Hughes Jones *et al.*, 1956; Gibson *et al.*, 1955), their results indicating an early loss of 2—10 per cent. Possible influences of the *in vitro* procedures are discussed above. It was shown by Hughes Jones & Mollison (1956) that the observed early loss was at least partially due to removal of tagged cells and not to an increased rate of Cr^{51}-elution. Reference is made to the most complete discussion presented in the latter paper (Hughes Jones *et al.*, 1956). From results obtained by tagging and transfusion of previously stored cells (Ebaugh *et al.*, 1953; Donohue *et al.*, 1955; Gibson *et al.*, 1955; Strumia, Taylor, Sample, Colwell & Dugan, 1955) it appears that the loss of activity during this phase indicates fairly well the removal of non-viable erythrocytes. However, at present minor changes in Cr^{51}-survival during the first 24 hours must be interpreted with caution due to the several possible factors influencing this part of the curve.

The complete curve. Cr^{51}-activity may be found in circulation up to or beyond 100 days, which could indicate that the potential life span of Cr^{51}-tagged cells can not be much shortened if at all (Eadie *et al.*, 1955). Usually, two data are given to characterize the slope. The first is the time after which 50 per cent of the Cr^{51}-activity contained in the transfused cells has disappeared. The other is the half-time for the random process of elution. Corrections for sampling are usually not included. The 50 per cent value is reported to be reached after 24 to 40 days with most mean values within 24—33 days (Ebaugh *et al.*, 1953; Necheles *et al.*, 1953; Weinstein *et al.*, 1954; Read, 1954; Read *et al.*, 1954; Sutherland *et al.*, 1954; Mollison *et al.*, 1955; Hollingsworth, 1955; Donohue *et al.*, 1955; Hughes Jones *et al.*, 1956; Joske, McAlister & Prankerd, 1956).

The apparently greater variation in the elution half-time with mean values ranging from 45 to 79 days is somewhat unclear (Ebaugh *et al.*, 1953; Weinstein *et al.*, 1954; Read *et al.*, 1954; Mollison *et al.*, 1955; Donohue *et al.*, 1955; Ebaugh *et al.*, 1955; Eadie *et al.*, 1955; Joske *et al.*, 1956; Hughes Jones *et al.*, 1956). This could partly be due to the method of calculation (Mollison *et al.*, 1955), the handling of the cells prior to and during infuson (see above), and whether changes in RCV during the period of observation were corrected for (Birkeland, 1958a).

The curve has been described as close to linear on semi-logarithmic paper up to 50 days (Read *et al.*, 1954; Donohue *et al.*, 1955; Necheles *et al.*, 1953; Joske *et al.*, 1956). Mollison *et al.* (1955) found the complete slope to be composed of an early com-

ent (half-period 1.5 days) and a slow one
lf-period 54.2 days). Gibson *et al.* (1955)
cate that the true disappearance curve is
posed of two and possibly three com-
ents: one initially very rapid correspond-
to removal of non-viable cells, followed
a slower decline up to 15—20 days, and an
n slower rate after 20 days.

Apart from the initial loss during the first
hours, the present control data in Fig. 1
$_0$ = 100 per cent) fall close to the theo-
cal curve (Equ. III) up to 45—50 days.
er that time a suggestion of a slower rate
disappearance may be present. In Fig. 2
$_0$ = 100 per cent, Equ. IV) such a sug-
tion is less obvious. The latter curve
u. IV) is considered to be a useful ap-
ximation to the true state from 24 hours
up to 50—60 days in the 10 healthy
ividuals.

CONCLUSION

A well standardized, gentle technic as de-
ibed above, utilizing 10 μg or less of
tallic Chromium per ml of red cells for
ubation, yields reproducible values in
lthy individuals. Loss of activity during
first 24 hours is greater than expected,
varies within relatively narrow limits.
e rate of Cr^{51}-elution from intact cells is
rly constant from 24 hours up to 50—60
s. This permits a conversion of Cr^{51}-
vival data to a form corresponding to the
ar aging process. The latter data fall
hin the range of generally accepted values
cell survival.

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A RED CELL BALANCE INDEX (B. I.)

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(Received for publication November 13, 1957)

is generally accepted that the total circting red cell mass (RCV) is maintained net balance between cell production and ruction, that is as a dynamic equilibrium. RCV does not change with time, the ation is described as a *steady state* (Cros- 1954): Production equals destruction, out stating anything about the absolute of the two processes.

(I) $RCV = p \times Tm$ (Fig. 1)

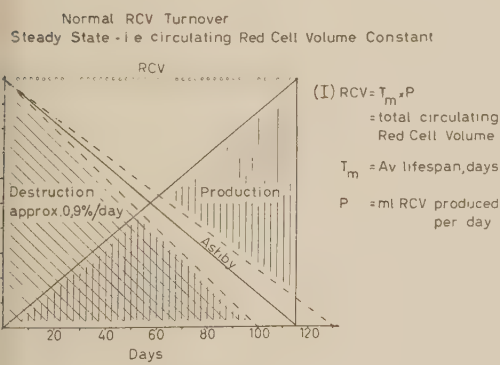


Fig. 1.

This study was conducted under U. S. Public lth Grant H-1142 as a Research Fellow in gery at the Medical College of Virginia, 1952—.

he preparation of the data was supported by rant from Nordisk Insulinfond, Gentofte, Denk.

where Tm = *average* life span of the red cells in days and p = ml of RCV produced per day on the average. Average daily destruction, $d = \frac{RCV}{Tm}$, and its absolute quantity expressed as ml of red cells destroyed per day is thus dependent on the existing RCV, as well as on the Tm.

The *steady state* is defined as *normal* when Tm is normal — *i. e.* Tm is 100 days or more ($Tm \geqq 100$). The RCV may increase or decrease with time, depending on whether the rate of production or of destruction dominates the picture. This is regarded as a *non-steady state* which is frequently encountered in various phases of trauma.

An attempt was made on a quantitative basis to express serial RCV-data obtained during non-steady state as deviations from the normal steady state by using a simple balance index (B. I.). As a record was kept of the amount of red cells removed for sampling and added as transfusions, the B. I. also permitted construction of a red cell balance diagram. The approach appeared to supply useful information during a study of the red cell turnover in thermal injury (Birkeland, 1958) and is reported separately in the present paper.

MATERIAL AND METHODS

The control subjects were healthy volunteers, 23—57 years old. The patients suffering from thermal injury were treated according to principles described by Evans (1952).

Wintrobe hematocrits were spun in duplicates under standardized conditions. The results were corrected for trapped plasma by using a factor determined to be 0.96 (Birkeland, 1958) and expressed as per cent (Hct). Hemoglobin was determined spectrophotometrically as oxyhemoglobin (Heilmeyer, 1933; Bell, Chambers & Wadell, 1945) and expressed as grams per 100 ml whole bood (Hgb). Mean corpuscular hemoglobin concentration (MCHC) was calculated as follows:

$$\text{MCHC} = \frac{\text{Hgb}}{\text{Hct}} \times 100$$

RCV was measured by using a modification of the P^{32}-method described by Reeve & Veall (1949), or it was calculated from plasma volume (PV) measured by using the T-1824 technic and a body hematocrit equal to 0.9 × Hct (Birkeland 1958) according to the formula

$$\text{Blood volume (BV)} = \frac{\text{PV}}{100 - 0.9 \times \text{Hct}} \times 100$$

$$\text{RCV} = \text{BV} - \text{PV}$$

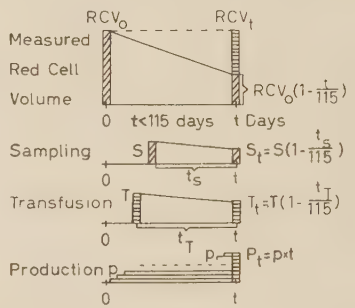
After injection of the tag material (P^{32}-cells or T-1824-solution) 3—6 venous samples were drawn without stasis at intervals during 30—60 min. Concentration was determined by extrapolation to zero time on a linear scale. — The quantities of cells removed and transfused were determined from volume and Hct. The quantities of viable cells in bank blood were estimated (Gibson, Peacock, Evans, Sack & Aub, 1947; Mollison, 1951) as follows:

No. of days stored	Per cent of viable cells
0— 7	100
8—14	90
15—18	80
19—21	70

Sampling between any two RCV-determinations averaged 26 ml of red cells (range 10—61 ml).

RCV was throughout expressed as ml of packed red cells. The packing was defined by the Hct.

Red Cell Volume Balance Index - B I



(II) $RCV_t = RCV_0(1-\frac{t}{115}) - S_t + T_t + P_t$

(III) $B.I. = \frac{p\times 100}{RCV_0} = \frac{P_t\times 100}{RCV_0\times t} = \frac{RCV_t - RCV_0(1-\frac{t}{115}) + S_t - T_t}{RCV_0\times t}\cdot 100$

Expressed as per cent per day of existing Red Cell Volume at Time 0.
For steady state B.I. = 0,87.

Fig. 2.

Derivation of B. I.

The B. I. was derived as shown diagrammatically in Fig. 2. RCV was measured at time zero (RCV_0) and on day t thereafter (RCV_t), where t is less than (<) 115 days. The latter figure was chosen as the average normal Tm (James III, Abbot, Norberg, Birkeland & Evans, 1953), assuming a normal red cell life span of 100—130 days (Crosby, 1954; Mollison, 1951; Ashby, 1948). At time t—t_S samples equal to S ml of red cells were removed, and at time t—t_T transfusions equal to T ml of viable cells were given.

The rate of destruction was assumed to be normal (Tm = 115 days) for all cells in the body. Of RCV_0 thus $RCV_0\ (1-\frac{t}{115})$ (Dornhorst, 1951) would have been left in the circulation at time t if no sampling had occurred. At the same time a quantity equal to S_t would have been left of S if the samples

e not removed. Of T only T_t would re-
n on day t.

he average daily production, p (ml red
released to the circulation), during t
s was assumed to accumulate because
e of the cells were destroyed ($t < 115$
s). The arithmetic sum of the assumed
itive and negative RCV-components at
e t will then be equal to RCV_t, Equ. II.
m Equ. II p may be expressed as per cent
RCV_0 per day as in Equ. III.

he resulting B. I. then expresses produc-
of red cells per day as percentage of the
ting red cell volume at time zero, assum-
a normal rate of destruction ($Tm \geq 100$
s). Corrections for sampling are included
B. I. During the normal steady state
V_0 is constant and $130 > Tm \geqq 100$)
range of B. I. will theoretically be $\frac{100}{Tm}$,
0.77—1.0. For $Tm = 115$ days B. I.
als 0.87.

The significance of the B. I.

3. I. within normal range indicates a
dy state without regard to true Tm.
f B. I. deviates significantly from the nor-
range, a *non steady state* is present. Tm
y be unknown. A positive deviation of
I. (B. I. > 1.0) indicates an increasing
V, *i. e.* a *positive red cell balance.* A nega-
e deviation of B. I. (B. I. < 0.77) means
ecreasing RCV, *i. e.* a *negative red cell*
ance.

f the B. I. deviates beyond certain limits,
inite conclusions may be arrived at re-
ding production and destruction of red
s (Equ. III):

3. I. > 1.0, then the net production is in-
ased ($p > \frac{RCV_0}{100}$), regardless of rate of
destruction. Whether the absolute value for p (expressed as ml/day) is above the accepted normal for an individual, depends on whether the measured RCV_0 is at or above the accepted normal for the individual concerned and also upon the true value of Tm.

0.77 > B. I. > 0, may be caused by a change in production, destruction, or both. Some production must be present ($p > O$).

B. I. = O, i. e. $RCV_t = RCV_0 \left(1 - \frac{t}{Tm}\right)$,

or theoretically $0.1 > B. I. > -0.13$, may be due either to a complete stop in production ($p = O$) with a destruction within normal range, or to an increased rate of destruction ($Tm < 100$) with a corresponding rate of production ($p > O$).

B. I. is negative, i. e.

$RCV_t < RCV_0 \left(1 - \frac{t}{100}\right)$ or *i.e.* B.I. < -0.13.

Then there is an increased destruction ($Tm < 100$), regardless of the rate of production.

If a complete record is kept of sampling and transfusions, periods of positive and negative red cell balance may be defined. Positive or negative balance may be expressed as ml of RCV:

$$\text{Balance} = \frac{(B. I. - 0.87) \times t \times RCV_0}{100}$$

To summarize: Periods of non-steady state may be quantitatively described as periods of positive and negative red cell balance in a practical balance diagram. When B. I.-values deviate beyond certain limits, periods of increased production or destruction may be ascertained without resorting to red cell survival studies. The different periods may be correlated with the clinical events in patients. The effects of these events on the red cell balance may be appreciated by inspection of the diagram.

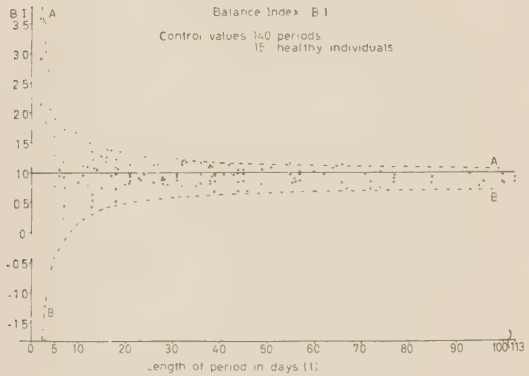


Fig. 3.

RESULTS

Control studies

Serial RCV-measurements were made in 15 healthy controls, totalling 140 balance periods of 2 to 113 days duration (Birkeland, 1958). The results are summarized in a scatter diagram (Fig. 3). The B. I.-values are given on the vertical axis, and the duration of the corresponding period (between RCV_o and RCV_t, *i. e.* O—t) is plotted in days on the horizontal axis. Values were calculated between any two RCV-determinations so that equal weight was given to each observation.

131 out of 140 values for B. I. in this control group fell within the range given by the theoretical (*i. e.* 0.77—1.0) B. I. $\pm\frac{6}{t}$.

The line A—A in the diagram shows the upper range: B. I. $= 1.0 + \frac{6}{t}$. The line B—B similarly shows the lower range: B. I. $= 0.77 - \frac{6}{t}$. A deviation beyond these limits is considered outside normal range.

The limit for negative B. I. can not be determined directly for obvious reasons. However, in accordance with the observed normal range it is assumed to be:

$$\text{B. I.} = -0.13 - \frac{6}{t}.$$

As can be seen the normal range of the B. I. decreases with the duration of the period between the two RCV-determinations used (RCV_o and RCV_t), *i. e.* with the duration of t. The relatively wide normal range (Fig. 3) results from errors in RCV-measurements and sampling (as reflected in $\frac{6}{t}$) as well as from physiological variations in rates of production and destruction (reflected in the range 0.77—1.0).

It may be noted from Fig. 3 that the B. I.-values tend to fall towards the upper limits for periods below 40 days. This tendency could in part be due to the repeated sampling which possibly stimulated production.

Clinical application

Below are given examples of the application of the B. I. in one blood donor and three patients. The diagrams were so constructed that the dotted horizontal line indicates the assumed normal values for RCV (males 28.5 and females 25.0 ml per kilo body weight), PV (45.5 ml/kg) as well as for BV.

O. R. means operation, and + 500 or 1000 indicate transfusion of one or two pints of blood respectively.

In Table I figures in brackets are assumed values. For red cell content of one blood bottle the assumed figure of 200 was based on testing of several bottles of bank blood (Birkeland, 1958).

B. Wal., blood donor (Fig. 4, Table I). T-1824 technic.

A healthy male, 34 years old, had 290 ml red cells removed by phlebotomy one day prior to the

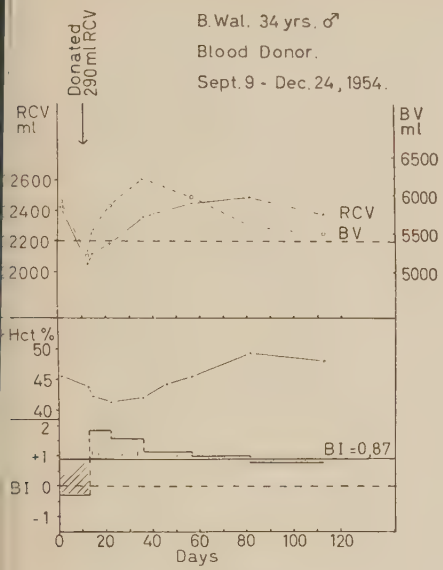


Fig. 4.

surement of RCV on day 13. When no cor-ion was made for the red cells removed by ebotomy, a B. I. equal to minus 0.3 was obtained. s value clearly indicates a non-steady state with egative red cell balance, and an increased de-ction is suggested. Quantitatively, this negative ance was equal to

$$\frac{(-0.3 - 0.87) \times 13 \times 2421}{100} = -368 \text{ ml RCV}$$

ich is a fair estimate of the amount removed. During the following two periods a non-steady te with positive red cell balance and increased duction is clearly indicated, showing the rege-ation of lost RCV. Quantitatively, the positive ance was equal to:

Day 13—22: 177 ml
Day 22—36: 205 »

Total: 382 ml

From day 36 on, a slight positive balance is gra-lly changing into equilibrium.

W. D., 53-year old male. Perforated gastric ulcer.

(Fig. 5, Table I). P^{32}-technic.

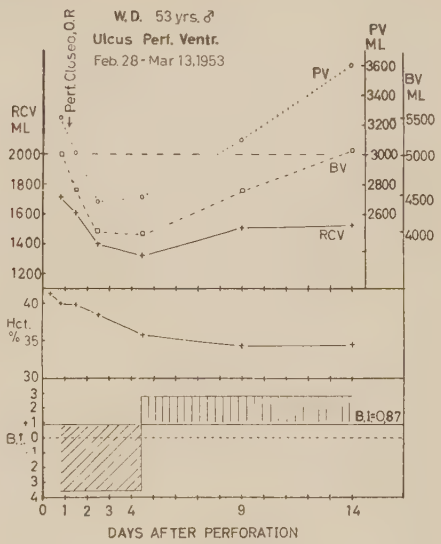


Fig. 5.

A non-steady state with negative balance and increased destruction is clearly borne out by a B. I. = —3.62 during the first 4 days. The rate of production was unknown. The trend of the RCV-data would indicate that the loss of red cells was not confined to the time of operation, but also took place during the following 3—4 days (Cfr. Fretheim, 1953).

Quantitatively, the negative balance amounted to —309 ml RCV.

During the following 9 days a non-steady state with positive red cell balance and increased production is shown. Rate of destruction was unknown, but likely to be normal. Quantitatively, the positive balance amounted to + 243 ml RCV.

The clinical course was free of complications.

P. A., 28-year old female. Burn, total 29 %, 25 % of 3°.

(Fig. 6, Table I). P^{32}-technic.

A non-steady state with negative red cell balance is present throughout, although not outside normal range during days 7—11. During the first 4 days an increased destruction is evident, and it is furthermore markedly so during days 11—17. The coincidence of pneumonia and negative red cell balance was observed in other patients (Birkeland, 1958).

Table 1. *Red cell balance following hemorrhage (B.Wal.), perforated gastric ulcer (W. D.) and thermal injury (P. A. and E. W.)*

Subject	Days	Body weight kg	Hct. per cent	RCV ml	PV ml	BV ml	MCHC	Sampling ml RCV	B. I.	Balance ml RCV
B. Wal.	0	72.6	45.4	2421	3503	5924	32.9			
Blood	12	Phlebotomy 290 ml RCV						-	—0.30	—368
donor	13	71.8	43.8	2052	3156	5208	-			
	15	71.8	42.2	2116	3452	5568	37.0	54	1.83	+177
	22	71.8	41.3	2181	3682	5863	37.8	25	1.54	+205
	36	69.5	42.1	2363	3871	6234	32.1	46	1.12	+124
	57	72.3	45.5	2452	3528	5980	-	34	0.98	+ 65
	81	67.7	49.3	2488	3115	5603	36.5	49	0.77	— 80
	113	66.4	48.0	2374	3122	5496	-			
W. D.	0	Perforation								
Ulcus	21 hrs.	-	41.0	1718	3247	4965	31.5			
perf.		O. R. Laparotomia. Perforation closed.						24		
ventric	36 »	67.7	40.9	1607*	3010	4617*	30.6	30	—3.62	—309
	2½	-	38.5	1403	2681	4084	38.7	32		
	4½	-	35.8	1325	2714	4039	33.0	21		
	9	68.0	34.4	1508	3101	4609	30.8		2.91	+243
	14	-	34.5	1532	3600	5132	29.6	20		
P. A.	0	Burned								
Burn	1 hr.	65	45.1	-	-	-	31.3			
29 %								18		
25 %	11 hrs.	-	40.3	1531	2369	3900	33.8	30	—5.32	—332
of 3°	4	-	30.5	1170	3111	4281	38.5	18	—1 28	— 75
	7	-	31.2	1077	2676	3753	33.1	17	0.35	— 22
	11	-	24.1	1038	3542	4580	31.6	24	—3.52	—273
	13	Transfusion		202						
	17	-	26.0	937	3055	3992	35.0			
	18	Transfusion		(400)						
	19	Deceased								
E. W.	0	Burned						20		
Burn	3 hrs.	70.0	40.1	1860	3400	5260	36.2			
25 %								32		
7 %	1	-	53.3	1747	1911	3658	33.4		—1.33	—368
of 3°								26		
	9	65.0	32.5	1439	3705	5144	38.9	20	1.26	+ 34
	15	-	37.0	1454	3211	4665	32.5	23	0.75	— 12
	22	60.0	37.8	1420	2491	3911	32.7			

Table I. (Cont.)

ect	Days	Body weight kg	Hct. per cent	RCV ml	PV ml	BV ml	MCHC	Sampling ml RCV	B. I.	Balance ml RCV
	28	O. R. Debridement. Transfusion		(400)				23	0.72	— 19
	31	55.9	45.7	1770	2716	4486	34.4			
	36	O. R. Skin grafting Transfusion		(200)						
	37	-	42.8	-	-	-	36.1	45	—0.55	—352
	42	Dressing off. Physiotherapy.								
	45	59.1	42.7	1562	2637	4199	35.1	26	1.56	+ 75
	52	60.2	41.7	1613	2736	4349	35.1	28	1.45	+178
	71	Discharged 65.8	40.3	1766	3412	5178	35.4	20	1.02	+ 90
	105	67.1	40.2	1843*	3253	5096*	34.6			

Obtained with the T-1824-technic.

The negative balance during the first 6½ days amounted to 407 ml, that is *26.6 % of the original RCV on day 1.* This patient may be taken as representative of a severe burn. The early phase with negative red cell balance of gradually decreasing magnitude is shown as well as the tendency to a relative increase in plasma volume with a total blood volume kept towards the normal level.

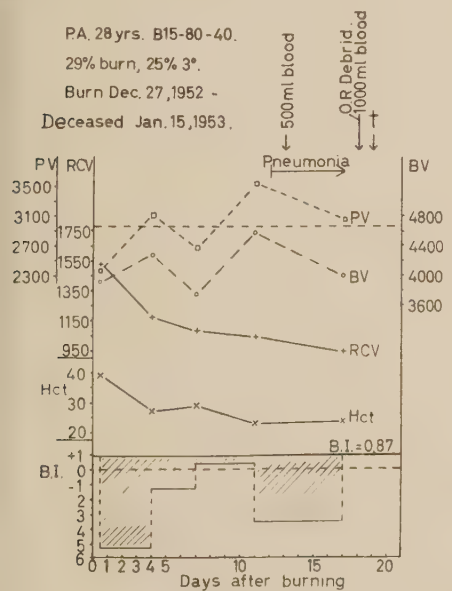


Fig. 6.

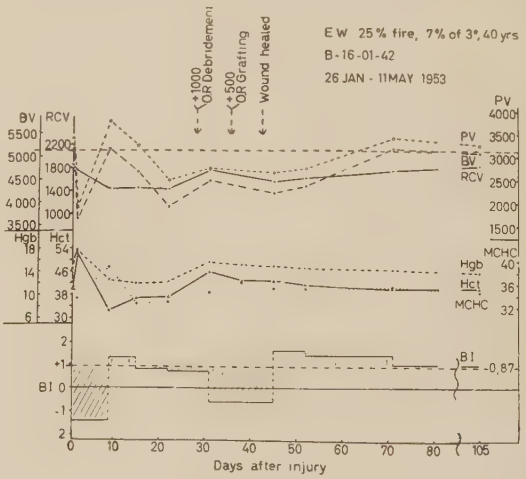


Fig. 7.

E. W., 40-year old male. Burn, total 25 % 7 % of 3°.

(Fig. 7, table I). P^{32}-technic.

The red cell balance diagram describes clearly the periods of positive and negative red cell balance as well as equilibrium in relation to the clinical events for this patient with a burn of moderate severity.

A non-steady state with a negative balance (total 368 ml RCV) and an increased destruction was present during the early phase following injury (first 9 days) as often observed in burns (Birkeland, 1958). During the following 22 days no significant deviation from the steady state was found, and the cell production was about normal (assuming a Tm within or near the normal range) or increased (if Tm was significantly shortened). A debridement did not cause great change. However, the skin-grafting procedure was most likely responsible for the next period of negative red cell balance (amounting to 352 ml) with an increased destruction (day 31—45).

It may be noted that the deficit was the same during the period of operation as it was after the first 9 days following injury when no external blood loss occurred.

During the 26 days following wound healing a positive red cell balance with an increased production was regenerating the RCV — a common observation in this phase (Birkeland, 1958).

DISCUSSION

Moore, Peacock, Blakeley & Cope (1946) introduced the concept of "red cell balance" as applied to burn patients. Their balance data were calculated as the average loss or gain of red cells per day after detailed adjustments were made for transfusions and for loss by dressing and operations.

The present approach is similar in principle. Corrections were made only for transfusions and samplings. External blood loss at operation *per se* was not separated from deficit in circulating red cell volume developing during the first post-operative days. From preliminary observations it was thought that the operative intervention could be directly or indirectly responsible for the total deficit during such a period (see W. D., Fig. 5).

It is emphasized that serial red cell volume data can not be properly evaluated without taking into consideration the turnover (production and destruction) of red cells as well as blood removal and transfusions. Data from patients are difficult to comprehend and to relate to clinical events unless they are presented as a balance diagram.

By applying the balance index, data are expressed in such a way that the rate of net change in circulating red cell volume with time may be compared in different individuals regardless of their size, sex and whether the existing red cell volume is normal.

Whether the change in balance is due to the clinical state *per se* (*e. g.* a raw surface area) or it is confined to the periods of operation may readily be seen from the chart, as in Fig. 6.

SUMMARY AND CONCLUSIONS

A red cell balance index (B. I.) and its application during red cell balance studies over a prolonged period of time is described. The B. I. expresses quantitatively the deviations from steady state among different individuals. Values for B. I. may be directly compared, regardless of individual differences with respect to total circulating red cell volume, body weight, age and sex.

Recording of the amount of red cells removed by sampling and added as transfusions as well as allowance for the normal aging process are mandatory for correct functional evaluation of red cell balance.

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HYPOTHERMIA AND INFECTION

I. INFLUENCE OF HYPOTHERMIA ON ANTIBODY FORMATIO IN MICE IN THE SECONDARY RESPONSE TO TYPHOID-H-ANTIGEN

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(Received for publication November 15, 1957)

Induced hypothermia has often been employed clinically in connection with infectious diseases. Nevertheless, experimental information on the influence of hypothermia on the host — parasite relationships in infectious conditions is by no means complete. The study presented here is a part of an experimental series aiming at a systematical investigation of hypothermia effects on different parts of the mechanism of infection.

Of previous investigations in this field only a study by Ipsen in 1952 will be mentioned here, as especially directed to the influence of hypothermia on active immunization. There it was shown that the protection of mice against tetanus toxin, effected by pre-immunization with the corresponding toxoid, was significantly decreased if the animals were kept at low temperature (+ 6° C) during the fortnight period of immunization. — It may be mentioned also that Gaiski (1944) found a disappearance or decrease of plague agglutinins and bacteriolysins during hibernation of marmots. A more comprehensive survey of the pertinent literature will be given in a following paper.

EXPERIMENTAL

Design of the experiments

Of common experimental animals, only su small ones as mice could be subjected to close controlled and uniform hypothermia in sufficient large numbers, desirable with consideration to t individual variation of antibody responses to an genic stimuli (Kohn, 1951; Berglund, 1956 a). T typhoid-H-antigen-antibody system was chosen presenting simple immunization technique and an body assay methods requiring very small seru volumes. For blood sampling and antibody titratio methods were employed which permitted up to subsequent tests from every mouse.

As a reasonably deep hypothermia could not maintained for more than 48 hours without losi a good deal of the animals, it was necessary to stu a rapidly displayed antibody reaction. This w achieved by initiating a secondary response wi a very large booster dose given to pre-immuniz animals, as done by Fagraeus (1948).

About 50 days after pre-immunization was nished, the antibody titres of most animals had d creased sufficiently to permit the rise of titres the secondary response to manifest itself clear The optimal route of administration and dose antigen for the booster was then sought in a seri of preliminary experiments with small groups. Th resulted in the choice of a subcutaneous injecti of 20 000 millions of bacteria. After this dose t

increase occurred after an interval of about ours and was most intense during the following ours.

two separate experiments hypothermia was tained for 48 hours, first during the initial imilation period", second during the following duction period", with a view to the different ts of cortisone (Berglund, 1956 b) and irradia- (Kohn, 1951; Dixon, Talmage & Maurer,) in different stages of antibody formation. mothermic control animals were kept in paral- Agglutinin titres were followed in every animal.

MATERIAL AND METHODS

Animals

lbino mice weighing 15—20 g at the beginning he experiment were numbered serially. Picking animals for control of preimmunization (see w), as well as allotting animals to different rimental groups, was performed by randomi- on according to random number tables of er & Yates (1953). At the final experiment weight of the animals ranged from 25 to 43 g.

Antigen

ormalin (0.4 per cent)-killed S. typhi strain . 57, rich in H-antigen. The stock suspension in per cent saline solution was standardized with roughs-Wellcome opacity tubes to contain 2.25 · bacteria/ml. Dilutions were made up with 0.9 cent saline solution.

Pre-immunization

0 mice were injected subcutaneously with the owing doses contained in 0.05 ml:

1	6	10	20	24	31	
20	70	200	2 000	2 000	2 000	mill.

On days 17, 28, and 35 the serum content of -H-agglutinin was tested in groups of 5 mice. until day 35 was the degree of immunization sidered sufficient, the mean titre then being 0 (1/3200-1/100). Already in this connection it observed that a small proportion of animals ned refractory to the antigen, which will be arent also from the final experiments to be cribed below.

After immunization the animals were kept until the titres had in general decreased to values of or below 1/100 when measured in randomized groups of 5 mice. This state was observed on day 82.

Blood sampling

From a cut in the tail 0.05 ml blood was drawn into a syringe (tuberculin type) containing 1.2 ml saline solution giving a dilution of 1 : 25, which was immediately centrifuged. The supernatant was stored in the frozen state.

Anti-H-agglutinin titration

The 1 : 25 serum saline dilutions were serially twofold diluted in 0.5 ml volumes. Same volume of the antigen stock solution diluted 1 : 60 was added to each tube. After incubation for 18 hours at 37° C the reactions were read as usual, whereby the last tube presenting agglutination visible to the naked eye was taken as the titration endpoint. When agglutination could be detected with a 6 × magnifying lens in the following tube, however, the endpoint was considered to be situated between this tube and the preceding one.

Except for Table I, the titres are expressed as "titre values", in terms of doublings of the initial titre, the latter being denoted as zero. Titre 1 thus means twice the original titre, titre 2 means 4 times and titre 3 is 8 times the original one. If no agglutinins were observed in the sample drawn prior to the booster injection (titre < 70), this negative reaction was, for convenience, denoted as zero as representing a basic immunity not detectable by the method employed. In this case titre 70 was considered as "titre 0.5", titre 100 as "titre 1.0", and so forth.

All titres ranged between $< 1/70$ and 1/18 000.

Hypothermia technique

After numerous previous experiments the following procedure was adopted as causing the least amount of stress during cooling down and warming up and permitting the highest survival percentage in connection with fairly prolonged (48 hours) and deep (22° C) hypothermia.

The experiment is started by a subcutaneous injection of 32 mcg/g body weight of Chlorpromazine-

HCl [1], followed after 30 minutes by an intraperitoneal injection of half this dose of ethyl-(1-methylbutyl)-malonylcarbamide-Na [2]. The mice are then, in their narcotic state, fixed with tape onto lead plates and submerged in the dorsal position into a thermostatically controlled 22° C water bath, only the head and the upper-anterior part of the thorax remaining above the water surface. Under these conditions rectal temperature varies between 21.5° and 23.5° C, the temperature in the lower esophagus ranging 0.5° higher. Oxygen is administered continuously to the cooling chamber formed by the water bath and its fairly tightly closed cover.

Warming up after 48 hours is begun by raising the water temperature slowly to 33° C, the period of warming being 4—5 hours. The animals then begin to show activity and are freed from their lead plates, placed into their original containers, and transferred into an incubator, the temperature of which is kept at 37° C for the first 1½ hour, thereafter at 31° C for 24 hours. Humidity is kept around 50 per cent and oxygen administration is continued. After this period the animals can be kept under normal conditions.

"Normothermic" control animals are given the same narcosis, whereby their rectal temperature decreases by 2—5° C, but are immediately subjected to the same warming up procedure described above.

Experiment with early hypothermia.

The influence on the antibody response of hypothermia, when covering a 48 hour period of the 60 hours generally passed before onset of the titre rise in normothermic animals, was studied in the first experiment. During this period the absorption of antigen takes place and its assimilation by the antibody forming apparatus is assumed to occur. It is called the *assimilation period* in the following.

In 13 mice hypothermia was induced 12 hours after the booster injection, while 9 normothermic controls were run in parallel. Blood samples were taken immediately before the booster injection (day 0 in the table and the graph), after 24 hours of hypothermia (day 2), in connection with the warming up after 48 hours of hypothermia (day 3), and thereafter 1, 2, 4, and, in two mice, 9 days after warming up (days 4, 5, 7 and 12 respectively). From the controls, samples were taken on the same occasions.

Experiment with late hypothermia

In another group of 13 mice, hypothermia was achieved for a 48 hour period starting 60 hours after the booster injection and covering the time of beginning titre increase in normothermic animals. This period is called the *production period* in the following. Six normothermic controls were run in parallel. Samples were taken immediately before the booster injection (day 0), in connection with induction of hypothermia (day 3), after 24 hours of hypothermia (day 4), at warming up after 48 hours (day 5), and thereafter 1, 2, 3, 5, 7, and 9 days after warming up (days 6, 7, 8, 10, 12, and 14 respectively). Samples from the controls were taken on the same occasions.

RESULTS

The titre curves of the control group in the first experiment and those of the same group in the second did not differ from each other to any significant degree. Both control groups are therefore reported together. The titres of the controls as well as of the early and of the late hypothermia groups are reported in Table I. In Fig. 1 the titre increases within the three groups are visualized more clearly by use of the "titre values" as defined above.

DISCUSSION

From Fig. 1 the following facts appear:

The antibody response was delayed by two days within both the hypothermic groups when compared with the controls, which time corresponds closely to the duration of hypothermia. Subsequently the titre increase of the animals subjected to hypothermia was as rapid as that of the controls. This observation

[1] Hibernal (AB Leo, Hälsingborg, Sweden).

[2] Nembutal (Abbott Laboratories, London, England).

ble I. *Titres of normothermic control (C), early hypothermia group (E), and late hypothermia group (L).*

Day	0	2	3	4	5	6	7	8	10	12	14
Titre											
1000										1	1
800							1	1			
000											
400									1	1	
500									2	2	
200						1	1	2			2
200					1	1	4				1
600					1		2			1	
100						1	2			1	
800					2						
560					1				1		
400				2	3				1	1	1
280					1		1	1			
200	1		1	2	1	1	1	1			
140	1	1		4							
100	4	3	5	1	1						
70		1									
<70	9	4	9	5	2	1	1				

Day	0	2	3	4	5	6	7	8	10	12	14
200		Hypo-					1			2	
200		ther-					1				
500		mia									
100					1		1				
800	1										
560	1	1	1	2	1						
400		1	1								
280											
200	1	1	1	1			2				
140					1						
100	3	1		1	1						
70	1	2	1	1	1						
<70	6	7	9	5	2						

Day	0	2	3	4	5	6	7	8	10	12	14
0 000										1	
5 400				Hypo-						1	2
4 500				ther-				1	2	2	1
3 200				mia					2	1	2
2 200							1	1	1		
1 600								2		1	
1 100							1	1	1		1
800							1	1			
560	1		1			1	2	1			
400						1	2				
280				1	1	2	1				
200						1					
140			1								
100	1			2	2	1					
70	2		2	4	2						
<70	9		8	5	5						

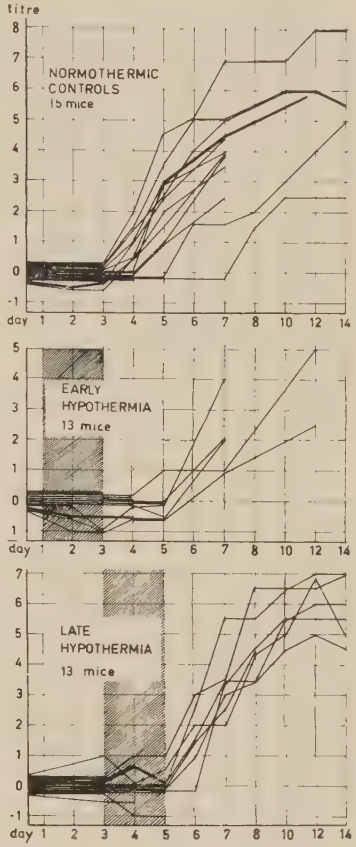

Fig. 1. Titre values of normothermic control group, early hypothermia group, and late hypothermia group. Initial titres are denoted as zero. Titre 1 means twice the initial titre, titre 2 four times, and titre 3 eight times the initial values, *etc.*

was statistically verified as will be seen in Table II. Here the titre values of the hypothermic animals for every day are compared with those of the controls by use of Student's t-test. The titre values of hypothermic animals were significantly lower during days 4 and 5, but not thereafter.

The delay was thus effected whether the hypothermia was achieved during the "assimilation period" or during the "production

Table II. *Statistical analysis of the titre values given in Table I.*

Day	Controls		Early hypothermia			Late hypothermia		
	n	M ± e(M)	n	M ± e(M)	P	n	M ± e(M)	P
2	9	–0.167 ±0.083	13	–0.192 ±0.090	>0.5			
3	15	–0.033 ±0.033	13	–0.308 ±0.121	>0.01* <0.05	12	0.045 ±0.097	>0.5
4	14	0.607 ±0.179	10	–0.150 ±0.076	>0.001** <0.01	12	0.083 ±0.149	>0.001** <0.01
5	13	2.115 ±0.377	7	–0.071 ±0.219	>0.001** <0.01	10	0.050 ±0.157	<0.001***
6	5	3.100 ±1.050				6	1.917 ±0.300	>0.01* <0.05
7	13	3.308 ±0.446	5	2.000 ±0.547	>0.05 <0.2	8	3.188 ±0.400	>0.05 <0.2
8	5	3.200 ±1.097				7	4.643 ±0.403	>0.05 <0.2
10	5	4.700 ±0.799				6	5.583 ±0.327	>0.2 <0.5
12	7	5.355 ±0.660	2	(3.750 ±1.263)	>0.2 <0.5	6	6.167 ±0.333	>0.2 <0.5
14	5	5.300 ±0.873				6	5.833 ±0.422	>0.5

n = number of observations M = arithmetic mean
e(M) = standard error of the mean P = probability

period", indicating that the assimilation of the antigen as well as the production of antibodies was inhibited in the hypothermic state. Whether this inhibition was due simply to decrease of cell metabolism during hypothermia or to hormonal or other disturbances connected with this condition cannot be established by these experiments. Whichever the mechanism may be, the effect is only a postponement, not an inhibition, of antibody formation.

Further, a tendency was noted toward depression of the antibody content on the two days following the booster injection (days 2 and 3). Such a depression could of course be observed only in animals found to have agglutinins before the booster. If the titre values of these animals are analysed separately, as done in Table III, the tendency is verified on certain points.

Thus, all values on day 2 taken together showed a statistically probable depression, normothermic animals not deviating from hypothermic ones. On day 3, however, only the titres of hypothermic animals were significantly below the initial values, thereby deviating from those of the normothermic animals, which had already returned to the initial level.

The titre depression is likely to be effected

Table III. *Statistical analysis of the titre values on days 2 and 3 of animals with demonstrable antibody before booster injection*

Group	n	M ± e(M)	P (diff. from zero)	P (mutual diff.)
Normothermic controls	5	−0.300 ±0.122	>0.05 <0.1	>0.1
Early hypothermia	7	−0.359 ±0.143	>0.05 <0.1	
Total, day 2	12	−0.333 ±0.134	>0.02 <0.05*	-
Normothermic controls + late hypothermia*	10	±0.000 ±0.129	-	>0.02 <0.05*
Early hypothermia	7	−0.591 ±0.171	>0.01 <0.02**	
Total, day 3	17	−0.235 ±0.123	>0.05 <0.1	-

*Up to day 3 the late hypothermic group is kept in normothermic condition.

neutralization of antibody by the very e antigen content of the booster injection. fact that the titres returned to the ori- l level in normothermic animals already lay 3 may be taken as a very first sign of body production observed in this group not in hypothermic animals.

SUMMARY AND CONCLUSIONS

or a study of the effect of hypothermia antibody formation, the rapidly displayed ondary response to a large booster dose Typhoid-H-antigen in white mice pre- nunized with the same antigen was chosen a convenient system for this purpose. ut one-third of the animals served as nothermic controls, one-third were sub- ed to a controlled hypothermia for the days following the booster (days 2 and 3, y hypothermia), and one-third were simi- y treated during the next two days (days d 5, late hypothermia). Hypothermia was eved by Hibernal + Nembutal + sub- sion into a 22° C water bath. Rectal tem- ture varied around 22° C. After hypo- thermia the animals were warmed up by an elaborate procedure, affording a fairly high percentage of survival. During hypothermia and for about one week thereafter, repeated blood samples were taken from each mouse, by cutting the tail, and individually tested for agglutinins.

The agglutinin titre increase following the booster injection was very rapid in all animals, but its onset was delayed by two days in both the hypothermic groups. Already before the start on day 4 of titre increase in the normothermic controls a difference was noted, in as much as an initial titre depression, most likely due to antibody neutralization by the large amount of antigen in the booster dose, was ended on day 3 in normothermic animals, whereas the depression was observed also on that day in the hypothermic groups.

It is concluded that the hypothermic state inhibits the assimilation of antigen (early hypothermia) as well as the production of antibody (late hypothermia). Inhibition is not absolute but effects only a postponement of the antibody response.

This inhibition or, more correctly, delay of antigen uptake and antibody production is considered to be important with a view to experimental as well as clinical application of the hypothermia technique in connection with infections.

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A QUANTITATIVE METHOD FOR ESTIMATION OF SOME CARBOHYDRATES ON FILTER PAPER

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(Received for publication November 20, 1957)

ince Partridge (1948) introduced the lication of partition chromatography on er for the separation of carbohydrates, a nber of different methods for the quanti- ve determination of the carbohydrate con- of isolated spots have been worked out. eview of these methods has recently been n by Kowkabany (1954). It appears n this review that elution of the spots owed by colorimetric or titrimetric deter- ation of the carbohydrates generally are methods to be preferred.

n the present paper an attempt has been le to work out a method for colorimetric ermination of some simple carbohydrates ng an aniline reagent 5 times more con- trated than the one used by White & ss (1956). In order to localize the carbo- rates on one as well as two-dimensional er chromatograms, the reaction is perfor- l on the paper before elution.

EXPERIMENTAL

Assay procedure.

lucose, galactose, lactose, xylose and arabinose e investigated. The spots to be analyzed contain g—1000 μg of carbohydrate, covering an area etween 4 cm²—16 cm² on Whatman No. 1 r paper. A strong light or a dark screen is ed behind the paper which is sprayed with aniline reagent until it just becomes translucent. The aniline reagent is prepared by mixing equal parts of 1.10 M alcoholic aniline with 1 M aqueous citric acid. The stock solutions are stored at 3—4° C. The aniline used is distilled *in vacuo*. The paper is dried completely at room temperature (18—23° C, relative humidity 50—70 per cent) in an air current. In our laboratory a drying time of 20—30 min. is used. The dry Whatman paper is placed in a metal container, 35 cm high and with an inner diameter of 14 cm, taking care that it is in close contact with the wall. The metal container is closed by a rubber stopper and is placed in a boiling water bath for exactly 10 min. The upper edge of the Whatman paper in the metal container must be below the surface of the water in the water bath. The carbohydrates will be seen as brown spots, the pentoses somewhat more red than the hexoses. The spots, and blanks of the same paper size, are cut out and placed in test tubes containing 10 ml n-butanol-pyridine-water (2:2:1). The test tubes are closed by rubber stoppers, which, however, after having been used several times may be attacked by the elution solvent and therefore often have to be renewed. The samples are eluated for 5 hours in the test tubes which are turned up and down five times every hour, and subsequently the samples are allowed to stand for 15 min. in order to sediment paper particles present in the solution. By use of a Beckman spectrophotometer model DU, the absorbance of the eluate at 420 mμ is determined against a reference consisting of the elution solvent.

The number of μg of one carbohydrate is calculated from the formula: $\frac{E \times 100}{\alpha \times I_c}$. E = absorb-

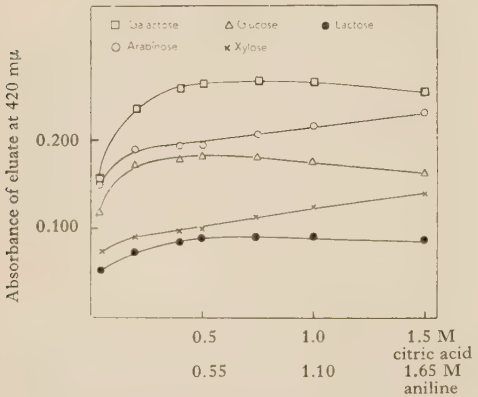

Fig. 1. Effect of concentration of aniline reagent.

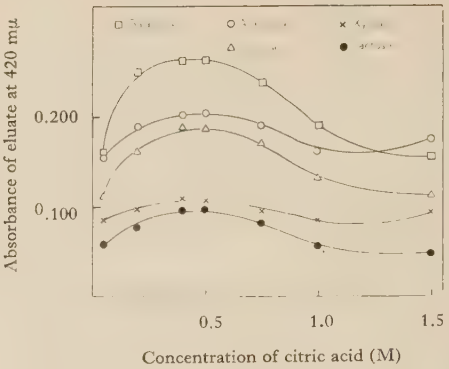

Fig. 2. Effect of citric acid concentration in the aniline reagent on color yield.

ance of eluate, deducted the average of 3 blanks. $\alpha = E^{0.01\,0/00}_{1\,cm}$ of galactose, calculated from the absorbance of two spots containing 125 μg galactose, which are treated and read together with the spots of unknown carbohydrate content. I_c is the color yield of the carbohydrate relative to galactose on μg basis.

Remarks on the different steps of the method

1) *The reagent.* White & Hess (1956) used a reagent consisting of a 1:1 mixture of 2 per cent (= 0.22 M) aniline in ethanol (v/v) and 0.2 M aqueous citric acid for the color development. In experiments with reagents containing different amounts of citric acid and aniline (Fig. 1) it was found that reagents more concentrated than the one used by White & Hess gave higher absorbances.

Fig. 2 gives the results of an experiment which was carried out in order to determine the *optimal concentration of citric acid.* Reagents were prepared which contained 0.55 M aniline and different concentrations of citric acid in 48 per cent ethanol. Maximal absorbance was found with the reagent containing 0.5 M citric acid.

The reagent containing 0.55 M aniline and 0.5 M citric acid in 48 per cent ethanol was chosen for further experiments.

2) In order to find the *optimal time of drying* of the paper sprayed with the aniline reagent, six pieces of Whatman No. 1 filter paper with spots containing 125 μg galactose, glucose, lactose or arabinose or 62.5 μg xylose were sprayed with the aniline reagent and placed in an air current at room temperature (20—21° C, relative humidity 70—78 per cent) for different periods and thereafter treated as described in the assay procedure. It appears from Fig. 3 that maximal color yield was obtained when the time of drying was 20 min or more. The experiment indicates that incomplete drying of the paper lowers the color yield. A time of drying of 20—30 min. was chosen under the conditions in the author's laboratory.

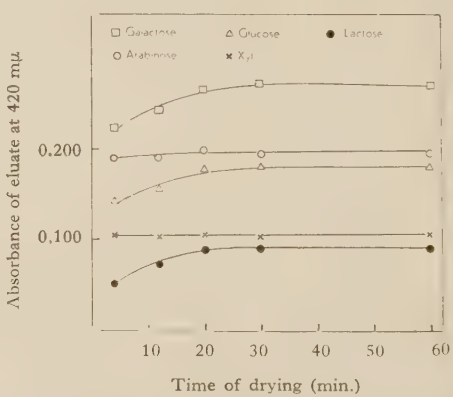

Fig. 3. Effect of time of drying on color development.

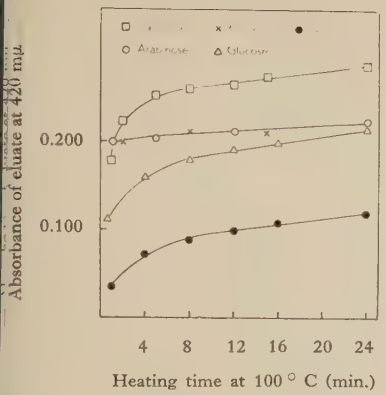

Fig. 4. Effect of heating time on color yield.

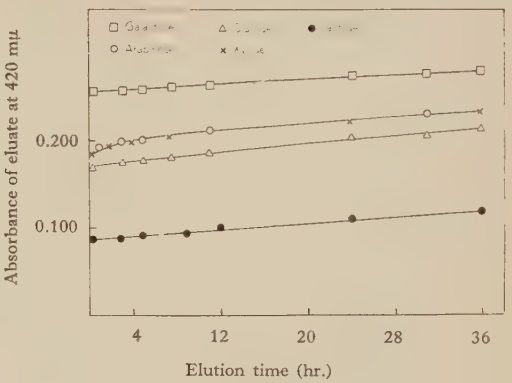

Fig. 5. Effect of elution time on color yield.

3) *Heating time.* Heating in a boiling water bath s chosen, as this procedure gave reproducible ults. In order to investigate the effect of heating e, spots containing 125 μg of the carbohydrates re placed on Whatman paper, sprayed with ani- reagent and heated in a boiling water bath m 1—24 min. It appears from Fig. 4 that the or yield of galactose, glucose and lactose in- ases very much with time when the heating time below 8 min. After that the rate of increase is ch lower. A heating time of 10 min. was pre- red in the assay procedure.

4) Choice of *the elution solvent.* A number of vents have been examined, among these ethanol- ter (1:1), toluene, benzene, xylene, n-butanol, idine and water, and a mixture of the three last ntioned fluids in the proportion 2:2:1. Spots containing 125 μg glucose, galactose, xylose or arabinose or 250 μg lactose, treated according to the assay procedure, were eluated for 5 hours with the solvents mentioned. It appears from Table I that none of the solvents gave maximum absorbance for all of the carbohydrates investigated. N-butanol-pyridine-water (2:2:1) was chosen as the best compromise.

5) In order to investigate the *effect of the elution time* on color yield, spots containing 125 μg glucose, galactose, lactose, arabinose or xylose were treated with the aniline reagent and read in the spectrophotometer after different elution periods. It appears from Fig. 5 that the absorbance increases only very slowly with time when the elution time is more than 3 hours. An elution time of 5 hours was chosen for practical reasons.

Table I. *Effect of various elution solvents.*

ution solvent	Absorbance at 420 mμ of eluates					
	Glucose	Galactose	Xylose	Arabinose	Lactose	Sum
nzene	0.005	0.003	0.009	0.009	0.001	0.027
luene	0.012	0.005	0.011	0.009	0.001	0.038
lene	0.018	0.027	0.021	0.025	0.010	0.101
ter	0.100	0.099	0.073	0.062	0.096	0.430
butanol	0.102	0.190	0.173	0.179	0.099	0.743
anol-water (1:1) ..	0.177	0.272	0.183	0.184	0.167	0.983
ridine	0.149	0.244	0.238	0.218	0.145	0.994
butanol-pyridine-water 2:2:1)	0.171	0.251	0.206	0.206	0.198	1.032

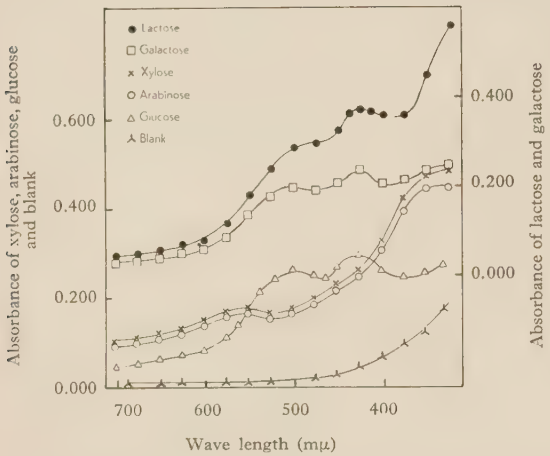

Fig. 6. Spectral curves of some carbohydrates coloured with aniline reagent.

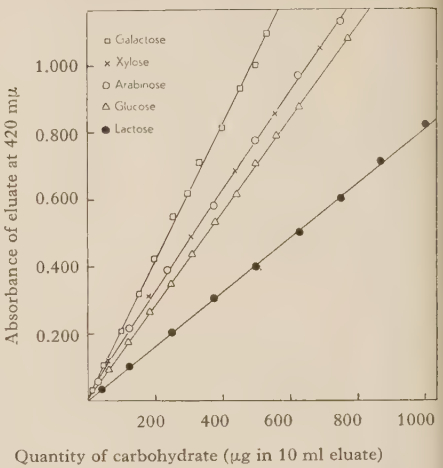

Fig. 7. Standard curves of the five carbohydrates.

6) To find a *wave length suited for reading the absorbance* of the eluate, spectral curves for the 5 carbohydrates investigated were read on a Beckman quartz recording spectrophotometer, model DR.

It is seen from Fig. 6 that there is a peak about 420 mμ for the hexoses. The pentoses show a small peak at 550 mμ, but because the extinction at 550 mμ is relatively small, it was chosen to read all the eluates at 420 mμ.

Relationship between amount of carbohydrate and absorbance

Spots containing 30—750 μg of glucose, xylose or arabinose, 15—550 μg of galactose or 40—1000 μg of lactose were treated according to the assay procedure. The relationship between absorbance of eluate and quantity of carbohydrate appears from Fig. 7.

It will be seen that the curve for xylose is identical with that for arabinose. Proportionality between quantity of carbohydrate and extinction was demonstrated for galactose, glucose and lactose. The standard curves of xylose and arabinose did not show complete proportionally between quantity of carbohydrate and extinction, but an approximation to proportionality was found in the range 100 μg—500 μg. It was decided to consider the curve as linear in this range. The curves were reproducible to within a few per cent. On the basis of the extinctions given in Fig. 7 the $E_{1\ cm}^{0.01\ \%}$ of each compound was calculated: glucose 0.141, galactose 0.205, lactose 0.081, xylose 0.158 and arabinose 0.157.

The color yield of the carbohydrates on μg basis relative to galactose (I_c) could not be calculated from the curves given in Fig. 7, as they were made on different days. A special experiment was carried out in order to determine the I_c values. Spots containing 125 μg, 250 μg and 500 μg glucose, arabinose and xylose, 250 μg, 500 μg and 1000 μg lactose and 125 μg galactose were determined at the same time. The determinations were repeated 3 times. The I_c values calculated on the basis of these experiments appear from Table II.

ble II. *Color yield of glucose, lactose, se and arabinose on μg basis relative to galactose*

ohydrate	Relative color yield (I_c)			
	1st determination	2 determination	3 determination	Mean
ucose	0.67	0.67	0.67	0.67
ctose	0.39	0.37	0.38	0.38
lose	0.70	0.71	0.71	0.71
abinose	0.70	0.71	0.71	0.71

he results of four *recovery experiments* spots containing known amounts of the ohydrates investigated, are summarized able III. The determinations were made ifferent days. In general the recovery of hexoses was found to be within 6 per of the expected value. The average re-ery of the 125 μg pentose spots was a higher than 100 per cent, and the re-ery of the 500 μg pentose spots a little er than the expected value.

o ascertain whether it is possible to de-nine small amounts of *carbohydrate in -containing solutions,* 1.5 ml of a 0.33 M ium acetate buffer (pH 5.48), containing 2 mg lactose, xylose, arabinose, glucose and galactose, was analyzed. In order to get a satisfying separation on the paper chromatograms, the sample was desalted on a 75 ml ion-exchange bed, consisting of a mixture of the cation-exchanger Zeocarb 225 treated with 3 M HCl and the anion-exchanger Amberlite IRA 400 treated with 2 N NaOH and 4 per cent acetic acid.

The sample was washed through the column using distilled water. 50 ml was collected, concentrated to dryness *in vacuo* and transferred quantitatively to a small glass tube with a diameter of 1.5 cm, using 3 + 2 + 1 + 1 ml distilled water and dried again *in vacuo* to permit dissolution of the carbohydrates in only 200 μl distilled water, containing 0.25 per cent benzoic acid to prevent fermentation. Three spots, each containing 50 μl, were placed on Whatman No. 1 filter paper and the carbohydrates were separated by descending one-dimensional paper chromatography for 48 hours, using n-butanol-pyridine-benzene-water (5:3:1:3) as solvent system (De Whalley, Albon & Gross, 1951).

It is seen from Table IV that the average recovery was within 8 per cent of the expected value.

a b l e III. *Four recovery experiments, made on different days*

ohydrate	Quantity added (μg)	Recovery in per cent	S. D. in per cent
ose	125	101	5
	500	102	4
ose	250	98	3
	1000	100	2
se	125	104	3
	500	97	3
inose	125	102	3
	500	97	2

Table IV. *Recovery of carbohydrates in a salt containing solution*

Compound	Quantity added (μg)	Quantity recovered (μg)	Recovered in per cent
Xylose	495	502	101
Arabinose	495	475	96
Glucose	495	494	100
Galactose	495	456	92
Lactose	495	458	93

DISCUSSION

Wallenfels, Bernt & Limberg (1953) treat carbohydrate spots on paper using a triphenyltetrazolium chloride reagent, whereafter the spots are eluated and colorimetrically determined. Fischer & Dörfel (1954), having investigated the triphenyltetrazolium chloride method, point out the importance of uniform heating and state that it is necessary to run a standard solution parallel to the unknown sample. The standard solution must contain the same carbohydrates in about the same quantity as the sample. This probably implies that the triphenyltetrazolium chloride method is rather time-consuming and more complicated than the present method.

In comparison with the methods in which elution of carbohydrate from the paper is performed before the color reaction, treating the carbohydrates on the paper with the color reagent has some advantages. The spots can be localized with greater certainty, and the method may therefore be used for quantitative estimation of spots on two-dimensional paper chromatograms. It appears from a subsequent paper that the method is well-suited for analysis of biological fluids containing a more complex mixture of carbohydrates.

SUMMARY

A method for quantitative estimation o glucose, galactose, lactose, xylose and ara binose on paper chromatograms is described The sugars are treated on the paper wit an aniline-citric acid reagent, eluated, and th optical density is read in a spectrophotomete at 420 mμ.

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QUANTITATIVE DETERMINATION OF SOME CARBOHYDRATES IN NORMAL URINE

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(Received for publication November 20, 1957)

t is well known that there are a number lifferent carbohydrates in normal urine. us, Harding, Nicholson & Archibald 36), using differential fermentation, nd glucose, galactose and other hexoses, Nicholson & Archibald (1939), using same method, found evidence suggesting presence of small amounts of pentoses urine. Eastham (1949), Montreuil & anger (1953), Futterman & Roe (1955) White & Hess (1956), using paper omatography, have identified various bohydrates in urine.

n the investigation reported here urine m 10 normal persons were de-ionized and centrated 25—65 times, whereafter the bohydrates were separated by two-dimenal paper chromatography. The carbodrate content of the isolated spots identi- as glucose, galactose, lactose, xylose and binose were determined by a colorimetric thod.

MATERIALS AND METHODS

Collection of urines

liquots of 24-hour urine specimens were obed from 5 healthy women ranging in age from o 34 years and 5 healthy men 24—31 years old, Table I. All the urines were preserved with ml of toluene and stored at —20° C pending ysis. Fehling's and Heller's tests were negative

T a b l e I. *Data for the subjects investigated*

Name (and sex)	Age (years)	Body weight (kg)	Height (cm)
K. J. (m)	24	60	167
P. H. M. (m)	25	61	170
P. J. C. (m)	31	75	187
J. N. (m)	24	82	191
J. D. (m)	29	69	176
G. N. (f)	24	53	167
H. B. (f)	18	66	176
I. A. (f)	18	63	169
I. S. (f)	34	50	169
E. M. A. (f)	22	58	170

in all cases. Food intakes were recorded for each 24-hour period and for the 6 hours preceding.

No attempt was made to control the diet except in one case (J. D.). For each of three 24-hour periods J. D. was given exactly the same diet, and for one 24-hour period J. D., having fasted overnight for 12 hours, got no food and nothing to drink except water.

All the subjects studied were doing their normal work during the period of collection of urines.

De-ionization and concentration of the urine

The urine samples for analysis were de-ionized and concentrated, because the presence of ions in the samples affects the separation of carbohydrates on paper chromatograms, and the concentration of carbohydrates is rather low in normal urines. Normally 8 ml aliquots were de-ionized. If the determination of specific gravity and volume indicated the specimen to be more concentrated or more dilute than normal, correspondingly smaller

or larger samples were de-ionized (between 5 and 13 ml). The urine was passed through a mixed-bed ion-exchanger column consisting of equal parts of Zeocarb 225 (H^+), and Amberlite IRA 400 (acetate) as described in a previous publication (Date, 1958), with the only modification that when the sample was washed through the column with distilled water, a quantity of 20 times the volume of the sample was collected. Finally the material was dissolved in 200 μl distilled water, containing 0.25 per cent benzoic acid to prevent fermentation.

Paper Chromatography

25—125 μl of the de-ionized, concentrated urine was placed as a spot (0.4 × 3.5 cm) on Whatman No. 1 filter paper (57.7 × 46.7 cm). The carbohydrates were separated by descending 2-dimensional paper chromatography, using the upper layer of n-butanol-pyridine-benzene-water (5 : 3 : 1 : 3) (Partridge, 1948) as solvent system for the first long-dimensional development, and either n-butanol-glacial acetic acid-water (4:1:5) (De Whalley, Albon & Gross, 1951) or ethyl acetate-glacial acetic acid-water (3:1:3) (Jermyn & Isherwood, 1949) as solvent system for the second short-dimensional development. The chromatogram was developed for 48 hours in the first and for 72 hours in the second solvent system.

Quantitative estimation of the carbohydrates

The paper chromatograms were dried completely after the development, sprayed with aniline reagent (0.55 M aniline and 0.5 M citric acid in 48 per cent ethanol) and placed in a metal container which was heated in a boiling water bath for 10 min. The spots were cut out, eluated and their carbohydrate content determined colorimetrically as described in a previous paper (Date, 1958). As it was not always possible to cut out spots and blanks of the same paper shape, their size was controlled by weighing.

The recovery of known quantities of carbohydrate added to 1.21 ml de-ionized urine (E. M. A.), which was placed on Whatman No. 1 filter paper, separated by two-dimensional paper chromatography and determined quantitatively, is seen from Table II. 170 μg of each carbohydrate was added to the urine. It appears that the recovery was within 8 per cent of the expected value.

Table II. *Recovery of carbohydrates added to a de-ionized urine sample*

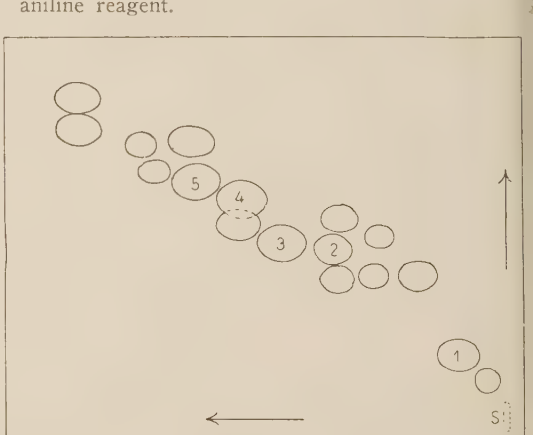
Carbohydrate	Amount of carbohydrate (μg) in the sample		Recovery in	
	with addition	without addition	μg	per cent
Xylose	444	287	157	92
Arabinose	320	147	173	102
Glucose	362	200	162	95
Galactose	227	68	159	94
Lactose	196	38	158	93

Identification of the carbohydrates

Fig. 1 shows a typical two-dimensional paper chromatogram of de-ionized urine (J. N.) colored with aniline reagent.

The spots of 1, 2, 3, 4, and 5 contained lactose, galactose, glucose, arabinose and xylose respectively. The methods used for the identification were:

1) Comparison with localization and color of known carbohydrates run parallel to the urine spots in one of the two developments. The pentoses were colored red-brown and the hexoses brown with aniline reagent.



First development: N-butanol-pyridine-benzene-water (5:3:1:3

Fig. 1. Typical two-dimensional paper chromatogram, colored with aniline reagent, of de-ionized urine from a normal young man. S = localization of urine spot before the development. 1 = lactose, 2 = galactose, 3 = glucose, 4 = arabinose and 5 = xylose.

ble III. *Melting point determinations of osazones prepared from urinary carbohydrates isolated by paper chromatography*

ound	Melting points (°C) of osazones from urine	of osazones prepared from pure carbohydrate	Values from literature	
osazone	204	205	200—208	Fischer (1908)
ctosazone	188	190	188—194	Fischer (1887 & 1908)
inosazone	160	163	163—164	Levene & Jacobs (1909)
sazone	158	159	159—160	Wohl & List (1897)
osazone	204	205	203—215	Fischer (1908)

Addition of carbohydrates to spots consisting e-ionized urine. The carbohydrates were sepa- by two-dimensional paper chromatography, ed with aniline reagent and compared with matograms without addition.

Re-chromatography of the spots in the fol- ng two systems: a) phenol-water (80:20, w/v) a HCN and NH_3 atmosphere (Consden, lon & Martin, 1944), b) ethyl acetate-pyridine- r (2:1:2, upper layer) (Jermyn & Isherwood,).

The spot containing lactose was submitted to hydrolysis and desalted. One-dimensional paper matography revealed that it consisted of equal s of glucose and galactose.

) Preparation of osazones. Glucosazone and sazone were identified by their typical micro- ic appearance. The melting points of the osa- s from urine were determined after repeated ystallisations in 30—40 per cent ethanol and pared with the melting point of osazones pre- d from pure carbohydrates, treated and deter- d together with the osazones from urine as mmended by Fischer (1908). The results are from Table III.

ecause it was impossible to isolate sufficiently quantities of carbohydrates from urine by dimensional paper chromatography for the e mentioned re-chromatography experiments or the preparation of osazones, a special experi- was carried out in a semipreparative scale. l urine samples were de-ionized using 400 ml xchange beds consisting of equal parts of Amberlite IRA 400 (acetate) and Zeocarb 225 (H^+). The de-ionized material was placed on Whatman No. 3 MM filter paper as a band (35 cm × 0.5 cm) and the carbohydrates were separated by one-dimensional paper chromatography twice in succession. As solvent system for the first development, n-butanol-pyridine-benzene-water (5:3:1:3) was used. Strips cut from different parts of the chromatogram were colored with aniline reagent to localize the carbohydrates, which were then eluated, concentrated to dryness *in vacuo* and placed again on Whatman paper. As solvent system for the second one-dimensional development, n-butanol-glacial acetic acid-water (4:1:5) was used. After drying of the chromatogram the carbohydrates were localized, eluated, concentrated to dryness and used for the identification experiments. In order to control the identity and purity of the carbohydrates isolated in semipreparative scale, a small amount of each was placed on Whatman No. 1 filter paper and analyzed by two-dimensional paper chromatography.

RESULTS

The concentration and the 24-hour excretions of the 5 carbohydrates investigated in the urines of 10 normal persons are given in Table IV. Glucose was generally present in the urines in larger quantity than any of the other carbohydrates, and the variation of the 24-hour excretion was considerable (56—

Table IV. *Concentration and 24-hour excretion of determinations of the concentration of each*

Name	Urine pH	Specific weight	Volume (ml)	Urinary N-excretion g/24 hr.	Xylose		Arabinose	
					μg/ml, ave.	mg/24 hr.	μg/ml, ave.	mg/24 hr.
K. J.	6	1.027	615	8.60	71, 74	73	45	45, 44	45	28
P. H. M.	6—7	1.020	1220	10.49	27, 28	28	34	33, 29	31	38
P. J. C.	7	1.022	1175	12.39	25, 23	24	28	21, 22	22	26
J. N.	6—7	1.023	1020	10.24	19, 20	20	20	12, 12	12	12
J. D.	6	1.016	1069	9.85	37, 44	41	44	47, 55	51	55
G. N.	6—7	1.017	1025	7.72	14, 14	14	14	20, 22	21	22
H. B.	6	1.029	593	8.44	126, 125	126	74	68, 61	65	39
I. A.	5.5	1.013	1092	8.52	91, 84	88	96	56, 46	51	56
I. S.	6.5	1.014	1138	8.19	18, 20	19	22	44, 40	42	48
E. M. A.	6	1.033	480	7.47	224, 237	231	111	112, 121	117	56
Average				9.19		66	49		46	38

542 mg/24 hr.). The excretion of arabinose and xylose was generally somewhat smaller than glucose. Only small amounts of galactose were excreted, whereas the quantity o lactose was somewhat larger. It is not pos sible from the results reported in Table IV

hydrates in normal human urine. Duplicate
hydrate investigated were carried out.

Glucose		Galactose			Lactose			Sum of the 5 carbohydrates (mg/24 hr.)	Remarks on the diet
l, ave.	mg/24 hr.	μg/ml	μg/ml, ave.	mg/24 hr.	μg/ml	μg/ml, ave.	mg/24 hr.		
882	542	24 23	24	15	43 41	42	26	656	A very large amount of carbohydrate in one single meal.
59	72	11 11	11	13	22 19	20	24	181	High carbohydrate intake, evenly distributed in the different meals
59	69	11 13	12	14	31 30	31	36	173	Small amounts of fruits
80	82	17 17	17	17	39 39	39	40	171	Small amounts of fruits
60	64	10 9	10	11	19 24	22	24	198	Medium contents of fruits, milk and carbohydrates
56	57	8 9	9	9	13 14	14	14	116	Small amounts of fruits, milk and carbohydrates
224	133	23 24	24	24	21 18	20	12	282	Large amounts of fruits (apples) and carbohydrates
120	130	16 14	15	16	21 18	20	22	319	Large amounts of carbohydrates
59	67	3 3	3	3	15 15	15	17	157	Small amounts of carbohydrates
165	79	48 56	52	25	26 31	29	14	285	Large amounts of fruits
176	130		18	14		25	23	254	

ecide if there is a difference in the carbo-
rate excretion of the two sexes. The
age excretion of lactose was consider-
ably higher in men than in women in the present material, but the intake of milk and milk products, roughly estimated from the

Table V. *Effect of diet on the excretion of some carbohydrates in urine. The person investigated (J. D.) was given the same diet for each of three 24-hour periods, while urines no. 1, 2 and 3 were collected. Urine no. 4 was collected during fasting. Duplicate determinations of the concentration of the carbohydrates investigated were carried out.*

Urine No.	Volume (ml)	N-excretion (g/24 hr.)	Xylose			Arabinose			Glucose			Galactose			Lactose		
			$\frac{\mu g}{ml}$	ave.	mg/24 hr.	$\frac{\mu g}{ml}$	ave.	mg/24 hr.	$\frac{\mu g}{ml}$	ave.	mg/24 hr.	$\frac{\mu g}{ml}$	ave.	mg/24 hr.	$\frac{\mu g}{ml}$	ave.	mg/24 hr.
1	1069	9.85	37 44	41	44	47 55	51	55	60 59	60	64	10 9	10	11	19 24	22	24
2	1215	10.28	41 43	42	51	47 49	48	58	60 58	59	72	6 7	7	9	21 20	21	26
3	1085	9.86	36 42	39	42	45 41	43	47	70 72	71	77	7 7	7	8	20 20	20	22
Average of 1, 2 and 3		10.00		41	46		47	53		63	71		8	9		21	24
4	1280	8.10	8 8	8	10	23 21	22	28	39 35	37	47	3 2	3	4	10 8	9	12

ry records, was about 30 per cent higher
en.

e relatively high excretion of glucose
J.'s urine is possibly due to a very high
e of carbohydrates in one single meal.
appears from Table V that the carbo-
ate excretion of one person (J. D.,
samples 1, 2 and 3) on different days
varied slightly when the diet given on
different days was the same. During
ng (J. D., urine sample 4) the 24 hour
tion of glucose decreased to 66 per cent,
tose to 44 per cent, lactose to 50 per
arabinose to 53 per cent and xylose to
er cent of the average values calculated
urine samples 1, 2, and 3.

DISCUSSION

is of interest to compare the chromato-
hic values given in Table IV with others.
ntitative determination of carbohydrates
rine using polarimetric or reduction
ods do not afford information about the
tity of the single carbohydrates. Deter-
tion of the fermentable substances in the
e is made by a number of authors after
us previous treatment of yeast or urine.
wirth (1922) finds an average excretion
rmentable substance in normal urine,
lated as glucose, of 334 mg/24 hr., Høst
3) finds on an average 368 mg/24 hr.,
West & Peterson (1932) 142 mg/24 hr.
excretion of glucose, which is assumed
e an essential part of the fermentable
ance of the urine, in the present investi-
n was averagely 130 mg/24 hr.

he use of paper chromatography for the
sis of carbohydrates has made it pos-
to separate even rather complex mix-
of these. Montreuil & Bolanger (1953), on the basis of one-dimensional paper chromatographic examinations of deionized urine samples, estimated the amount of excreted glucose, arabinose and xylose, to be between 30—50 μg/ml urine. In the present work the average excretion of glucose was somewhat higher and the variations in the excretion of all three carbohydrates were greater than estimated by Montreuil & Bolanger. Montreuil & Bolanger found only small quantities of galactose in the urine. They give no figures for the quantity of lactose in urine.

White & Hess (1956) separated the carbohydrates of normal urines on two-dimensional paper chromatograms. By comparison with the color intensities of spots containing known quantities of the various carbohydrates, they roughly estimated the amounts of each carbohydrate in urine. The normal range was from 25—100 μg/ml for the aldoses. The excretion of glucose, xylose and arabinose in the present investigation varied more than reported by White & Hess.

The origin of the carbohydrates in the urine is assumed to be either the carbohydrates resorbed from the intestine or the carbohydrates produced by the organism itself. Using methods of fermentation, Neuwirth (1922) and Harding & Selby (1931) demonstrated the influence of the supply of food on the excretion of carbohydrate in urine, and West, Lange & Peterson (1932) found decreased amounts of fermentable substance in the urines of persons during starvation. The present investigation confirms that the diet has a marked influence on the carbohydrate excretion in urine. It would generally be desirable to give a controlled diet in experiments where the carbohydrate excretion

of different groups of persons is to be compared.

No relationship was found in the present investigation between amount of carbohydrate and N-excretion in urine.

SUMMARY

The carbohydrate excretion in 24-hour urine collections from 5 normal young men and 5 normal young women was examined by help of two-dimensional paper chromatography. Quantitative values for glucose, galactose, lactose, xylose and arabinose, which were present in all the urine collections investigated, were obtained by a colorimetric method. Glucose was excreted in higher amounts than any of the other carbohydrates. More xylose and arabinose was excreted in urine than lactose. Only small amounts of galactose were generally present in urine.

Only small variations were found in the excretion of the carbohydrates when one person was given the same diet for three 24-hour periods. During fasting the excretion of all the carbohydrates investigated decreased considerably.

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IN VITRO STUDIES OF BACTERIAL SENSITIVITY TO TETRACYCLINE AND OLEANDOMYCIN IN COMBINATION*

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(Received for publication November 20, 1957)

The question of synergism and antagonism between different antibacterial agents aused great interest early in the history of antibiotics research. Thus Ungar (1943) demonstrated synergism between sulphonamides and penicillin. Jawetz (1952) and Jawetz, Gunnison, Coleman & Kempe (1955) recognized two groups of antibacterial drugs, one with bactericidal activity (penicillin, streptomycin, bacitracin, neomycin and polymyxin B), and the other bacteriostatic (tetracyclines, chloramphenicol, chloromycin and sulphonamides). These authors encountered synergism most commonly among drugs of the first group, occasionally when one of the second was added to an agent of the first, but not among drugs of the second group. Antagonism could occur between drugs from the two groups were combined.

The findings from *in vitro* studies seldom have practical clinical implications. Combined penicillin and streptomycin therapy in subacute ulcerous endocarditis caused by enterococci is usually cited to illustrate synergism. Antagonism of clinical importance has been demonstrated in penicillin treatment of pneumococcal meningitis. Otherwise synergism and antagonism between antibiotics were not attributed major practical significance until specific combinations of antibiotics, above all tetracycline and oleandomycin, became commercially available.

Synergism between tetracycline and oleandomycin was demonstrated *in vitro* by English, McBride, Halsema & Carlozzi (1956). Garrod (1957), however, failed to reproduce these results, although he used the same technique and also, to some extent, the same bacterial strains. From experiments on mice, English *et al.* reported similars synergism, but their technique was criticized in a leading article in the British Medical Journal (1957), and it would seem at least equally valid to interpret the results as being solely due to an additive effect. Attempts to make clinical use of antibiotic synergism were described by Carter & Maley (1957) and other writers. The value of specific antibiotic combinations and the practical significance of synergism have been vigorously discussed in the U.S.A. (Welch, 1957; Finland, 1957; Dowling, 1957), in England (Garrod, 1957), and in Scandinavia (Tunevall, 1957; Frisk, 1957; Klövstad, 1957).

To demonstrate synergism *in vitro*, English *et al.* (1956) tested broth cultures of bacteria against increasing concentrations of

* Aided by a grant from Pfizer AB.

tetracycline and oleandomycin separately, and against the same amount of the two drugs combined in the proportions used in the Pfizer preparation sigmamycin (PA 775, 67 per cent tetracycline and 33 per cent oleandomycin). This method, however, is too laborious for routine testing of bacterial sensitivity to combinations of antibiotics. We have therefore employed a modification of the method described by Elek, Hilson & Jewell (1953), which seems to be suitable for routine work with a wide variety of clinical isolates.

METHODS AND MATERIALS

Sensitivity tests were made with 335 bacterial strains, comprising *Micrococcus pyogenes* var. *aureus, Micrococcus pyogenes* var. *albus, Diplococcus pneumoniae, Streptoccous* α and β, *Pseudomonas aeruginosa, Alcaligenes faecalis, Escherichia coli, Aerobacter aerogenes, Proteus sp.*, enterococci and coliform types. These strains had been isolated during the routine work of the clinical bacteriologic laboratory from varied sources, mainly urine, pus and sputum from the wards and out-patient clinics. They were tested for sensitivity to tetracycline and oleandomycin separately and to the two drugs in combination.

The bacterial sensitivity to tetracycline and oleandomycin was initially determined with the aid of a paper disc method according to Ericsson, Högman & Wickman (1954). Each disc was impregnated with 50 mcg of one of the antibiotics. The zone of inhibition was read in mm. Strains with an inhibition zone not exceeding 10 mm were classified as drug resistant. This zone corresponded to a concentration of c. 32 mcg of tetracycline or 200 mcg of oleandomycin per ml. On blood agar plates inoculated as for the initial sensitivity tests, tetracycline and oleandomycin discs were placed at a distance equal to half the sum of the diameters of the previously determined zones of inhibition. Synergism, if it existed, could be expected to produce an increased effect in the tangential region of the two inhibition zones, while antagonism should lead to increased bacterial growth in this region.

These "primary plates" with the tetracycline and oleandomycin discs on either side of the calculated zone were incubated for 24 hours. "Replica plates" were then prepared from them according to the technique described by Lederberg & Lederberg (1952) for the selection of mutants. Replica plating was used by Elek *et al.* (1953) in routine work with combinations of antibiotics. The method involves transfer of bacterial colonies, or even solitary bacteria, from one substrate surface to another by means of an impressed velvet stamp, without altering the distribution of the bacteria. The antibiotic discs are removed from the primary plates before the velvet stamp is applied. From the replica plates may be read any bactericidal action of the individual antibiotics or increased bactericidal effect of combinations of the drugs. The primary plates, on the other hand, show only the inhibiting activity and do not indicate whether this is bactericidal or bacteriostatic.

In evaluating bacteriostatic or bactericidal effect according to the replica plating technique, we have used the classification of Manten (1956). If the inhibition zone on a primary plate developed no more than 5 bacterial colonies when transferred to a replica plate, bactericidal potency was ascribed to the antibiotic or combinations of antibiotics tested; 5 to 25 colonies indicated a partially bactericidal, and more than 25 colonies a bacteriostatic effect.

The strains upon which tetracycline and oleandomycin showed synergism in tests with the diffusion method were further assayed in liquid medium, to which the antibiotics were added singly in different concentrations or together in strengths and proportions varying according to a chess-board system. Five ml of meat-infusion broth at pH 72 were used and the inoculate was 0.1 ml of a 24-hour broth culture. Growth was read after incubation for 20 hours at 37° C.

RESULTS

Synergism between the antibiotics was demonstrated on 11 strains, of which 4 were staphylococci and 7 were *E. coli* or coliform bacteria.

Replica plates were prepared from 89 of the primary plates (Table I). The synergism

ble I. *The effect of tetracycline and oleandomycin on 89 bacterial strains determined with replica plates.*

Strain	Tetracycline					Oleandomycin			
	Number of strains	No* effect	Bacteriostatic effect	Partial bactericidal effect	Bactericidal effect	No* effect	Bacteriostatic effect	Partial bactericidal effect	Bactericidal effect
yogenes var. *aureus* ..	37	11	15	10	1		17	17	3
yogenes var. *albus*	13	7	5		1		9	3	1
mococci	1				1				1
ptococci	3		2		1		2		1
ptococci	3		2	1			2	1	
rococci	5		4		1		5		
ruginosa	1		1			1			
zecalis	2				2				2
li	18		6	9	3	18			
orm bact.	3		1	2		3			
rogenes	1			1		1			
rus sp.	2	1		1		2			
.....	89	19	36	24	10	25	35	21	8

* Diameter of inhibition zone on primary plates less than 10 mm.

Table II. *Chess-board testing of synergism.*

	Tetracycline mcg per ml.							
	0	0.05	0.1	0.2	0.4	0.8	1.6	3.2
	4+	4+	3+	2+	+	—	—	—
	4+	4+	3+	2+	+	—	—	—
	4+	4+	3+	2+	+	—	—	—
	4+	4+	3+	2+	+	—	—	—
	4+	3+	2+	+	+	—	—	—
	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—

Strain 209 *st. aureus* "No interaction".

Table III. *Chess-board testing of synergism.*

Oleandomycin mcg per ml.	Tetracycline mcg per ml.							
	0	0.5	1.0	2.0	4.0	8.0	16.0	32.0
0	4+	4+	4+	4+	4+	4+	4+	3+
0.5	4+	4+	4+	4+	4+	4+	3+	2+
1.0	4+	4+	4+	4+	4+	3+	2+	+
2.0	4+	4+	4+	4+	3+	2+	+	—
4.0	4+	3+	3+	2+	2+	+	—	—
8.0	3+	3+	3+	2+	+	+	—	—
16.0	2+	2+	+	+	—	—	—	—
32.0	+	+	—	—	—	—	—	—

Strain 043 *st. aureus* Synergism: 4+16 or 16+4.

n on the primary plates was reproduced the replica plates, but was more conspi- us with 1 *E. coli* and 1 coliform strain. ndomycin produced no inhibition zone n these 2 strains, but seemed to increase bactericidal action of the tetracycline.

Control tests according to a chess-board system of which examples are given in Tables II and III demonstrated that with 4 of the 11 strains this effect was true synergism, since the total amount of antibiotic required to produce inhibition was less when

Table IV. *Results of tests on synergism according to diffusion method and chess-board system.*

Bact. strain		Result on petridish	Minimal inhibiting dose mcg per ml:			Result
			Oleando.	Tetra.	Ol.+tetra.	
209 (Oxford)	*St. aureus*	No interaction	0.4	0.4	0.4 + 0.4	No interaction
043	*St. aureus*	Synergism	32	32	4 + 16 16 + 4	Synergism
586	*St. albus*	Synergism	1	32	0.5 + 2.0	False syn.
882	*St. aureus*	Synergism	3	32	2 + 0.5	Synergism
567	*St. albus*	Synergism	2	32	1.0 + 0.5	Synergism
890	Coliform	No interaction	32	4	* 4	No interaction
732	Coliform	Synergism	32	8	8 + 4	False syn.
742	*E. coli*	Synergism	32	16	4 + 2	Synergism
073	*E. coli*	Synergism	32	4	* 4	No interaction
047	*E. coli*	Synergism	32	8	* 8	No interaction
011	Coliform	Synergism	32	4	* 4	No interaction
063	*E. coli*	Synergism	32	2	* 2	No interaction
049	*E. coli*	Synergism	32	8	* 8	No interaction

* No effect of Oleandomycin.

oleandomycin and tetracycline were combined than when either drug was given separately. Two other strains (586 and 732) displayed some synergistic inhibition according to the chess-board system. Here the effect of the antibiotic with the weaker action (tetracycline on staphylococci and oleandomycin on bacteria of the coli group) was detecatble inasmuch as high dosage of this agent reduced the minimum inhibitory concentration of the more effective antibiotic. Assessment of the results according to the size of the total dose of antibiotic showed that on these 2 strains the effect of the more active agent alone was greater than that of the paired antibiotics. This we designated "false synergism".

To check the methods employed, tests were made of synergism between bacitracin and streptomycin. This was demonstrated both with the diffusion and with the chess-board system and was of the type "true synergism".

Two bacterial strains on which tetracycline and oleandomycin had shown no interaction when tested with the diffusion method gave similar negative results in chess-board tests.

DISCUSSION

Because of the indications which have been suggested for administration of tetracycline and oleandomycin in fixed combination, we considered it desirable to investigate if synergism could be demonstrated with the method used for routine tests of bacterial sensitivity in our laboratory (Ericsson, Högman, Wickman 1954). This method implies that the search for synergism is made with antibiotics in concentrations corresponding to their lowest bacteriostatic dose. "Replica plating" was added to show bactericidal action. Further, the effect of varying concentrations and proportions of the two antibiotics was tested according to a chess-board system.

ne results indicate that tetracycline and ndomycin synergistically inhibit 3 per of the tested bacterial strains when the biotics are used in concentrations corre- ding to their minimum inhibitory dose. tion tests showed that with all but one ne coli strains (742) the point at which rgism became operative (> 32 mcg per f tetracycline, 8 mcg of oleandomycin) far above the antibiotic concentrations h conceivably occur in a focus of infec- With strain 742 and with one strain) of staphylococci the synergism ap- ed at the upper limit of clinically ob- ble concentrations of oleandomycin — cg per ml. One staphylococcal strain) and one coliform (732) showed "false rgism" in that the total minimum in- ory dose of the combined antibiotics was er than the corresponding dose of the which was the more effective of the Only with three strains (043, 567 and was weak synergistic inhibition demon- le with clinically feasible concentrations ntibiotic.

hecks of the methods employed showed to be appropriate for the purposes of investigation.

SUMMARY AND CONCLUSIONS

vestigations of the joint effect *in vitro* eandomycin and tetracycline showed that drugs acted independently of each other 7 per cent of 335 mixed bacterial strains clinical sources. On the other strains synergism was demonstrable with cer- laboratory techniques. Only in 4 cases true synergism found, however, and in f these the minimum inhibitory concen- n of one antibiotic corresponded to the upper limit of clinically feasible dosage. While it is thus probable that oleandomycin and tetracycline may display synergism *in vitro,* our investigations do not suggest that this synergism has clinical value. It seems unlikely that the complicated mechanism of *in vitro* synergism has any clinical analogy *in vivo.*

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A ONE STAGE METHOD FOR THE ASSAY OF ANTIHEMOPHILIC FACTOR B (AHF B)

WITH A COMMENT ON THE ANTIHEMOPHILIC FACTOR B (AHF B) CONCENTRATION IN PHENYLINDANEDIONE (PID) THERAPY

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(Received for publication November 20, 1957)

Although there have been a number of reports on the effect of prothrombopenic anticoagulants on the antihemophilic factor B (AHF B) activity within the last years, there is still considerable disagreement on this subject. Part of the confusion has arisen from the fact that the tests used for its determination have either not been sufficiently sensitive or they have been too technical and time consuming. Different investigators using the same technique or a slight modification of it have arrived at entirely different conclusions.

The methods that have been used thus far are listed in reverse order of their sensitivity: Corrective effect by test plasma or whole blood on 1) the whole blood clotting time, 2) on recalcification time, 3) on the prothrombin consumption of AHF B deficient blood or plasma. 4) Correction of the thromboplastin generation test of AHF B deficient plasma with test serum, and 5) a modified thrombin generation test using "purified" blood clotting fractions as reagents. Although the techniques have improved, the method presented here is believed to be the simplest, least expensive, and probably one of the most sensitive tests thus far proposed.

The purpose of this investigation was to determine the influence of phenylindanedione (PID) on the AHF B level in blood with a sensitive, standardized method.

Walker & Hunter (1954) demonstrated a deficiency of a serum clotting factor which affected thromboplastin generation during administration of coumarin (Naphthylhydroxycoumarin). They concluded that this result was caused by a deficiency of either 1) Factor VII, 2) an additional factor in serum, *i. e.*, Koller's Factor X, or 3) Christmas factor. Deutsch (1956) also has shown that Marcoumar may affect the "blood thromboplastin" formation. Marx (1955) found that dicoumarol and dicoumarol derivatives caused a reduction in Factor IX (AHF B). Sise, Kimball & Adamis (1955) also demonstrated that PTC (AHF B) was reduced after "phenindione" and dicoumarol therapy, but this effect was not seen in all patients. Naeye (1956) found that the reduction of PTC (AHF B) in patients on dicoumarol and coumarin therapy was never lower than 20 per cent of normal and the lowest activity occurred after about 5 days of treatment.

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Douglas (1956) demonstrated that the antihemophilic globulin (AHF A) consumption was completely defective in Christmas disease (AHF B deficiency) and also defective although to a lesser degree in phenylindanedione (PID) therapy, indicating a decrease in AHF B. Johnson & Seegers (1956), using a type of thrombin generation test which utilized a purified prothrombin preparation as the substrate, found that Autoprothrombin II (AHF B) was reduced to zero levels with therapeutic doses of dicoumarol. In a later paper, Johnson (1957) found that Marcoumar had no significant effect on Autoprothrombin II (AHF B). In their most recent publication, Johnson, Seegers, Koppl & Olwin (1957) reported that Sintrom, phenylindanedione, and dicumarol had a marked depressive effect on Platelet Co-factor II (AHF B) and caused this factor to be reduced to zero limits in some cases, whereas, Dipaxin, Marcoumar, Tromexan, and Cyclocumarol had no significant effect on this factor. Verstraete & Vandenbroucke (1957) demonstrated that Christmas factor (AHF B) was decreased also by Tromexan and Marcoumar.

REAGENTS AND METHODS

Reagents

Citrated antihemophilic factor B deficient plasma was prepared by collecting blood from a patient with severe antihemophilic factor B deficiency. The blood was collected in a 500 ml siliconized centrifuge bottle containing 1/10 volume of 3.13 gm per cent sodium citrate dihydrate and initially centrifuged in a refrigerated centrifuge for 60 minutes at 2500 rpm to remove the red cells and most of the platelets. The plasma was then transferred to lusteroid tubes and centrifuged at 20,000 rpm in an ultracentrifuge for 60 minutes to remove the remainder of the platelets and to enable the separation of the clear plasma from the plasma fat which might hinder the detection of end points. The plasma was then collected from beneath the fatty layer by means of a siliconized glass pipette and placed in lusteroid tubes to be stoppered and stored at —20° C in suitable aliquots until needed. Thus far such plasma has held up for several months without appreciable deterioration or change. It is not advisable to refreeze this reagent for re-use once it has been thawed, although no great changes have been noted in doing so. This reagent supplies, presumely, all the necessary coagulation factors except AHF B and platelets.

Cephalin suspension 1/200 in veronal buffer was prepared according to Hjort's technique (1955) from a crude acetone-insoluble, ether-soluble fraction of human brain, by dissolving 0.05 ml of the stock reagent in 9.95 ml of veronal buffer. This concentration has been found to be the optimum concentration of cephalin for "cephalin times" by Dr. Waaler in this laboratory. Cephalin suspension probably only supplies a reagent that is similar to the platelet lipoid factor (platelet factor 3).

Veronal buffer with an ionic strength of 0.154 and pH 7.35 was made as described by Owren (1947, p. 125).

Adsorbed-dialyzed ox plasma. The adsorbed ox plasma was prepared according to the technique of Hjort *et al.* (1955) and in addition it was dialyzed against 0.9 per cent sodium chloride solution, and had the pH adjusted to 7.3. The adsorbed-dialyzed ox plasma was kept in small stoppered glass tubes at —20° C. This reagent may be kept for months at —20° C without appreciable deterioration. It presumedly supplies an excess of AHF A, antihemophilic factor C, proaccelerin, and fibrinogen.

Calcium chloride 0.03 M

Dilution fluid I. (Hjort *et al.*, 1955) — a dilution fluid with the same ionic strength and concentration of anticoagulant as plasma from blood with a normal hematocrit, was prepared by mixing 100 ml of 3.13 gm per cent sodium citrate dihydrate (or 100 ml of 2.5 gm per cent potassium oxalate monohydrate) with 500 ml of 0.9 gm per cent sodium chloride solution.

Dilution fluid II. (Hjort *et al.*, 1955) — a dilution fluid with a pH 7.35, ionic strength 0.154, and

table low concentration of anticoagulant, was red by mixing 200 ml of veronal buffer, 200 5.66 mM sodium citrate (or 150 ml 51.3 mM sium oxalate) and 0.9 gm per cent sodium ide solution of sufficient quantity to measure ml.

Methods

The prothrombin-proconvertin (P-P) method wren & Aas (1951).

Specific prothrombin method: the RVV in alin method of Hjort *et al.* (1955).

Proconvertin assay: The method of Aas, plasma from a patient with congenital hypo-nvertinemia as substrate (1952).

Antihemophilic factor B assay (AHF B) (see below).

inciple of the Assay of Antihemophilic factor B (AHF B)

he principle involved in this one stage method ssay for AHF B is essentially the same as for of Langdell and co-workers' method of assay antihemophilic factor A (1953), in which they onstrated that "partial thromboplastins" (ce-in) clotted hemophilic plasma slower than nal plasma. This defect could be quantitatively ected in a graduated fashion by adding normal ma in gradually increasing quantities. This is true for AHF B deficient plasma which also a prolonged "partial thromboplastin" time halin time). In the assay method presented all blood clotting factors (except AHF B) in either optimum concentration, or in a stan-ized excess. The AHF B deficient plasma, h is used as a substrate, is normal in all cts except for its deficiency of this factor. adsorbed-dialyzed ox plasma provides an ex-of antihemophilic factor A, antihemophilic r C, proaccelerin, and fibrinogen. The cephalin ension substitutes for platelet factor 3, which t otherwise have been variable. Therefore the source of AHF B is from the plasma to be d.

1/20 dilution of the plasma to be tested for F B activity is used. Higher concentrations of plasma give clotting times which do not vary enough between the dilutions to be graduated satisfactorily in a dilution curve. Greater dilutions of the test plasma give clotting times that are too prolonged. Plasma is preferred to serum or stored serum because there is less danger of interference from known and unknown serum accelerators and also because we do not know the exact relationship between plasma AHF B activity and serum AHF B activity. The AHF B activity of the test plasma may be expressed as per cent of normal when compared to a dilution curve made from pooled plasma from a number of normal individuals.

Procedure

The blood to be tested is collected by silicone technique in a siliconized centrifuge tube, and centrifuged at 2500 rpm for 30 minutes to remove the red blood cells and most of the platelets. The tube is stoppered and kept at 4° C and tested as soon as possible. The plasma is first diluted 1/20 with dilution fluid II in a lusteroid tube. The tube is covered with a small square of parafilm and inverted 10 times to insure a thorough mixing. Experiments have shown that if the first dilution is made in lusteroid tubes shortly after the blood has been drawn, the dilution is quite stable, showing no change in the AHF B activity during 4 to 5 hours. If, however, the plasma is allowed to stand in contact with glass and in contact with platelets under ordinary conditions of room temperature, there may be a considerable shortening of the clotting time. It is therefore recommended, that until all of the variables with regard to handling the blood specimens are known, one should carry out the test as outlined above.

The test is done by mixing 0.2 ml of the 1/20 diluted test plasma, 0.2 ml of citrated AHF B deficient plasma, 0.1 ml of adsorbed-dialyzed ox plasma and 0.2 ml of the 1/200 cephalin suspension. This mixture is incubated for 6 minutes in a water bath at 37° C. At that time, 0.2 ml of the 0.03 M calcium chloride solution is blown into the small glass test tubes. Simultaneously a stop-watch is started. A standardized mixing is provided by tilting the test tube 3 times before replacing it into the water bath for final incubation. The end-point, which is the first formation of small fibrin threads

which usually are attached to the test tube wall near the surface of the mixture, is best seen with a cross light against a dark background. The small fibrin threads can best be seen by half-rotations of the test tube. There is an interval of several seconds (approximately 10—15 seconds when a 1/20 dilution of normal plasma is used) between the formation of the first fibrin threads and the following cloudy translucency and the formation of a solid clot. Although the translucent cloud could be an easier end-point to see, it is more unreliable and not as reproducable. The use of new, clean test tubes greatly facilitates the reading of the end-point.

It is further recommended to run all tests in triplicates, using the first tube to find the approximate clotting time, and the second and third to find the shorter and more correct clotting time. Since agitation may break up the first fibrin threads and give a false prolonged clotting time, it is best to leave the second and third tubes in the water bath undisturbed until just a few seconds before the clotting time, which was determined with the first tube.

RESULTS

Preparation of the correlation graph and selection of the dilution of the test plasma

The AHF B standardization curve or correlation graph was prepared by using serial dilutions of pooled plasma from several normal individuals. A dilution of 1/20 of this pooled plasma in dilution fluid II was arbitrarily designated as 100 per cent. Serial dilutions were made in two steps as shown in Table I, using dilution fluids I and II to keep a constant concentration of anticoagulant in all samples.

The AHF B concentration of the test plasma is read from the correlation graph, as illustrated in Fig. 1, which relates the clotting time to the AHF B content of dilutions of a standard plasma. The times, when plotted on double logarithmic paper, produce a straight line which bends at approximately

Table I. *Serial dilutions used in the preparation of the correlation graph for the assay of antihemophilic factor B (AHF B assay).*

Per cent concentration	Dilution	Primary dilution (P. D.)		Secondary dilution	
		Plasma ml	Dilution fluid I ml	P. D. ml	Dilution fluid II ml
100	1/20	0.2	—	—	3.8
50	1/40	0.2	0.2	0.2	3.8
33.3	1/60	0.2	0.4	0.2	3.8
20	1/100	0.2	0.8	0.2	3.8
10	1/200	0.2	1.8	0.2	3.8

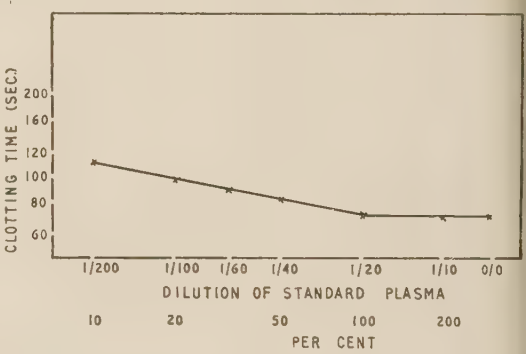


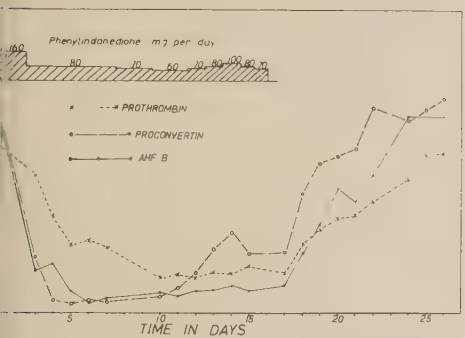
Fig. 1. Correlation graph obtained by plotting on a double logarithmic scale the clotting time of the AHF B assay system by testing dilutions of a standard plasma.

AHF B assay system: 0.2 ml AHF B deficient plasma, 0.2 ml test plasma dilution, 0.1 ml adsorbed-dialyzed ox plasma reagent, 0.2 ml cephalin suspension 1/200, and 0.2 ml 30 mM $CaCl_2$.

71 seconds (a dilution of 1/20 of the normal plasma). If the test plasma has a great deficiency of AHF B, it may be advisable to use a lesser dilution, *i. e.*, 1/10 dilution, in order to shorten the clotting time. Correction can then be made with the necessary calculations.

le II. *Antihemophilic factor B assay normal individuals chosen at random.*

plasma	Clotting time in seconds	Per cent concentration
. H.	71.0	110.0
. H.	71.5	105.0
. W.	72.0	100.0
I. S.	72.5	98.0
. A.	72.5	98.0
. T.	73.5	94.0
. T. A.	73.0	96.0
). H.	72.0	100.0
V. P.	72.5	98.0
. S.	71.5	105.0



2. The effect of treatment with phenylindane- (PID) on prothrombin, proconvertin, and HF B plasma levels in a normal individual.

he standard plasma in this case was pre- d by pooling plasma from three nor- individuals after the plasmas had been cted by silicone technique and spun free latelets. The standard plasma may be ed in stoppered lusteroid tubes at —20°C used as the normal control when desired. ore correct 100 per cent normal value be obtained by averaging the values a larger number of normals. The esti- d AHF B concentrations in normals within a range of aproximately 20 per Table II shows the results obtained 10 normal individuals.

Table III. *Antihemophilic factor B content of various types of plasma.*

Type of test plasma	Clotting time in seconds	Per cent concentration
1) AHF B deficient plasma	207.0	0
2) Normal plasma	72.0	100.0
3) AHF B deficient patient 24 hours after a transfusion (pt. I. O. M.)	109.0	11.0
4) Idiopatic thrombocytopenic purpura (platelets 20,000/c.mm. of blood)	71.5	105.0
5) Proconvertin deficient plasma	71.5	105.0
6) Long term treatment PID plasma (pt. J. W.)	91.5	27.0
7) Short term treatment PID plasma (pt. R. H.)	110.5	10.5
8) AHF A deficient plasma (pt. R. J.)	71.0	110.0

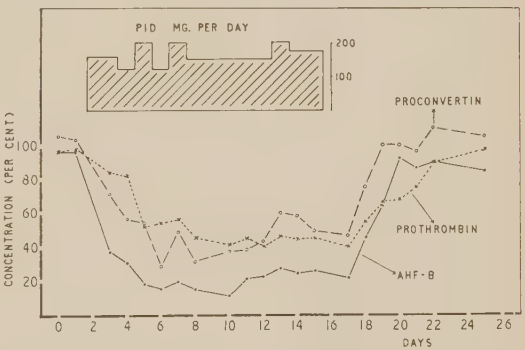


Fig. 3. The effect of treatment with phenylindanedione (PID) on prothrombin, proconvertin, and AHF B plasma levels in a normal person with a lower sensitivity than in Fig. 2. Note that AHF B plasma levels remained below levels of prothrombin and proconvertin during the treatment period.

Examples

In order partly to demonstrate the specificity of the assay system for AHF B, a number of different types of plasma were tested at random. The results are shown in Table III.

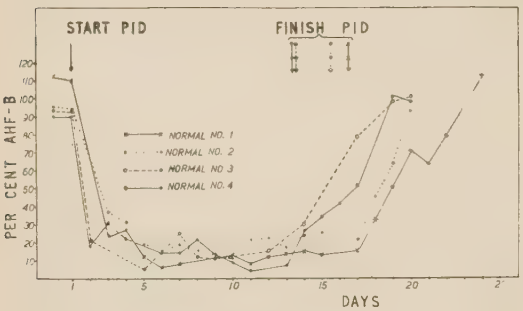


Fig. 4. The parallel effects of PID in therapeutic doses on AHF B in four normals.

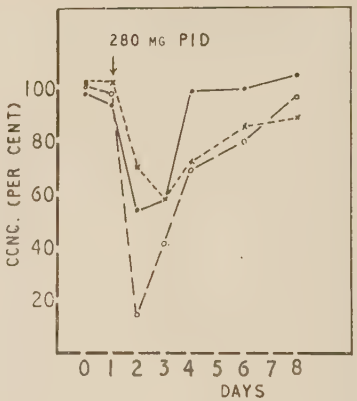


Fig. 5. The effects of a single test dose of PID (280 mg) on prothrombin, proconvertin, and AHF B blood levels in a normal person.
Prothrombin - - - x - - - x - - -
Proconvertin —— o ——
AHF B —— • —— • ——

Determination of the effect of phenylindanedione therapy (PID) on antihemophilic factor B (AHF B) activity as shown by the AHF B assay

Parallel studies were made on AHF B, prothrombin and proconvertin. The P-P test (prothrombin-proconvertin test) was also done, but is not recorded on the graphs. The P-P determinations, which also reflect changes in Stuart factor and AHF B, usually showed an apparent composite effect of the change in the three factors tested for. The determinations for AHF B were done by one of us (W. F. S.), the other tests by another person (S. M.).

Fig. 2 (pt. R. H.) shows the results of a study on one of 4 normal volunteers and Fig. 3 (pt. B. W.) the results obtained on the least sensitive of the four. The results obtained on the remaining two closely paralleled those seen in Fig. 2. The parallel effects on AHF B in the four normals can be seen in Fig. 4.

The effect of PID on AHF B is evident within 24 hours and plasma levels seem to follow more closely those of proconvertin than of prothrombin. Complete recovery to normal blood levels of AHF B after discontinuation of treatment took from 3 to 6 days.

Fig. 5 (pt. S. M.) shows an example of the typical effects of a single dose (280 mg) of PID. The plasma level of AHF B on a single dose in this study became less reduced than that of proconvertin.

A certain difference in the effects of short term PID therapy (2 weeks) as compared to long term therapy was found by comparison of the P-P values, prothrombin, proconvertin, and AHF B values of a group of patients from each of these categories.

None of the patients on short term therapy had a P-P value below the values of all of the three other factors tested for. As mentioned earlier, the P-P values were equivalent to an apparent composite of the values of prothrombin, proconvertin, and AHF B. On long term therapy, however, 8 out of 11 cases which were chosen at random showed P-P values which were lower than all of the

e factors tested. This difference may be ed to the behavior of the Stuart factor h also influences the P-P time.

DISCUSSION

his study confirms the findings of Marx 55), Sise *et al.* (1955), Douglas (1956), Johnson *et al.* (1956, 1957). We did not h the zero levels that Johnson *et al.* 57 b) observed, but sometimes we found ls below 10 per cent. Contrary to the ings of Johnson *et al.* (1957 b), our ies revealed a recovery of AHF B levels in 3 to 5 days, whereas in their studies d levels of Platelet Co-factor II (AHF still showed great reduction 10 to 12 after withdrawal of PID. The explana- very probably is the fact that maximal t as well as recovery time depend on of drug, dosage and sensitivity of the ent. The lowest levels of AHF B con- ration were usually reached after 3 to 5 s of treatment, to a certain extent depend- upon dosage and sensitivity.

ne reason to believe that a reduction of hemophilic factors is important in anti- gulant therapy lies in the fact that hemo- iacs have an extremely low incidence of cardial infarction. Only two cases of rosclerosis (one with myocardial infarc-) in hemophiliacs have been reported in the last four years (Boivin, 1954; wart & Acheson, 1957). Both of these s were males past the age of 70.

ne fact brought out by some of the re- investigations is that of the two most l tests for the control of oral anticoa- nts, the P and P test but not the original ck one stage method of prothrombin de- termination, reflects changes in the AHF B activity. This is best shown by Waaler's experiments (1957) demonstrating the simultaneous contribution to the formation of thrombin by the intrinsic and extrinsic clotting systems, and the influence of both systems on the P and P values. The P and P test is affected not only by prothrombin and proconvertin, but also by AHF B (especially at low P and P values), and Stuart factor.

AHF B is considered by some workers (Fantl & Sawers, 1954) to play a similar role in the intrinsic system as proconvertin in the extrinsic system of clotting. It may be more than a mere coincidence that AHF B and proconvertin levels seem to follow one another during anticoagulant treatment. Bell & Alton (1955) have reported a case of combined deficiency of Christmas disease (AHF B deficiency) and Factor VII deficiency (hypoproconvertinemia).

All factors depressed on oral anticoagulants respond to vitamin K_1 therapy.

SUMMARY

From a brief review of the literature, the recent experimental results that have led to the concept that antihemophilic factor B may play a more important role in anticoagulant therapy, are presented.

Many of the earlier findings have been confirmed and further experimental proof has shown that antihemophilic factor B (AHF B) is decreased during therapy with phenylindanedione (PID).

A one stage method for the assay of antihemophilic factor B (AHF B assay) has been presented along with experimental findings of parallel studies made on AHF B, prothrombin, proconvertin, and P-P values

of patients on long term, short term (2 weeks) and test dose (1 day) PID therapy. These results are correlated as to similarities and differences. Conclusions were that AHF B seems to follow proconvertin blood levels during the course of therapy. The effect of a single dose of PID, insofar as AHF B was concerned, was short lived (3—5 days).

ACKNOWLEDGMENT

I wish to express my appreciation to Professor P. A. Owren, the staff of Medical Department A, Rikshospitalet, and especially to my colleague Dr. Bjarne Waaler, without whose help and timely criticisms much of the above work could not have been completed.

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A METHOD FOR THE DETERMINATION OF TRYPSIN INHIBITORS IN URINE AND SERUM

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(Received for publication December 3, 1957)

he presence in urine of a specific sub-
e with antitryptic activity was recog-
l many years ago. The first extensive
cal investigations into the variation in
ary excretion of this inhibitor are due to
rd (1950). Astrup & Sterndorff (1955)
d the inhibitory effect to be specifically
ted against trypsin while other pro-
tic enzymes were not affected. As the
ods employed by Dillard and other in-
gators cannot be considered truly quanti-
e, the author has felt justified in describ-
n assay method that is easy to perform
t the same time satisfactory with regard
s degree of accuracy. A similar proce-
based on the same principles can be
for the determination of trypsin inhi-
in serum. Since it is simpler than the
nt methods employed for this purpose
ll also be discussed briefly.

METHOD

Principle

oteolytic activity is determined by the measure-
of the absorbancy at 280 mμ of the hydro-
products remaining in solution after precipita-
f a casein digest with trichloracetic acid. By
se of a curve depicting the ultraviolet absorp-
as a function of enzyme concentration, the
ity of enzyme inhibited by a sample added to
wn amount of enzyme is estimated. As urine contains large amounts of material having a considerable absorbancy at 280 mμ, only a small volume of urine can be assayed. Consequently the method must permit the determination of very small quantities of trypsin. It has proved possible to devise a procedure that involves a minimum of operations while satisfying the above conditions.

Reagents

Casein solution. Six grams of casein according to Hammarsten was weighed into a 100 ml volumetric flask and suspended by vigorous shaking with about 50 ml distilled water. Afterwards 4 ml 1 *N* NaOH was added. The protein was left to dissolve at room temperature overnight. On the following day water (saturated with toluene) was added to make a 100 ml volume. This stock solution was stored in the refrigerator; it was not used for more than a month.

Substrate casein solution was prepared from 10 ml stock solution diluted to 50 ml (1.2 g per 100 ml) with the phosphate buffer described below.

Phosphate buffer according to Sørensen. A mixture of 38.9 ml $\frac{1}{15}$ *M* primary potassium phosphate, and 61.1 ml $\frac{1}{15}$ *M* secondary sodium phosphate pH 7.00

Trichloracetic acid solution. It was 10 per cent $\left(\frac{W}{V}\right)$ or 0.61 *M*.

Trypsin. A crystalline preparation manufactured by "Novo terapeutisk Laboratorium", Copenhagen, was used. Its water content was determined by dry-

ing at 100° C *in vacuo* over phosphorous pentoxide in an Abderhalden drying pistol.

A stock solution was prepared by dissolving a quantity of the undried powder containing 20 mg dry matter in 10 ml 0.0025 *N* HCl. This solution was stable for about two weeks in the refrigerator. On the day of the experiment a dilute solution was prepared: For urine assays, 0.30 ml of the stock solution was diluted to 25 ml with 0.0025 *N* HCl. Of this solution, 0.25 ml contained 6 μg trypsin. For serum assays, 0.25 ml was diluted to 10 ml, of which 0.25 ml contained 12.5 μg trypsin.

Procedure

A. Determination of trypsin inhibitor in urine. The reaction was carried out in centrifuge tubes in each of which was placed 0.25 ml of the enzyme solution containing 6 μg trypsin. The tube was incubated in a water bath at 30° C, after which 1 ml diluted urine (see below) was added. A glass rod was used for stirring and was left in the tube. Exactly 15 minutes after addition of the urine, 2 ml of the 1.2 per cent casein solution was added. Digestion was allowed to proceed for 65 minutes. After this, undigested protein was precipitated by addition of 3 ml of the 10 per cent trichloracetic acid solution. At least thirty minutes later the tube was centrifuged for 10 minutes at 2000 r.p.m. The supernatant was poured directly into the photometer cuvette. A blank was run simultaneously and in the same way as the sample with the exception that the trypsin was added after the trichloracetic acid.

As mentioned above, urine contains considerable amounts of substances absorbing in the ultraviolet range. Therefore it is particularly important to measure the volume (1 ml) of diluted urine very carefully so that there is exactly the same quantity in the blank as in the sample.

The difference ΔE between the absorbancies (read against those of the blanks) of a standard and of a sample was of course an expression of the quantity of trypsin inhibited.

The urine was tested after dilution with the phosphate buffer. The ratio of dilution used was 1 : 20, 1 : 100, or 1 : 200 according to the inhibitor content. In certain cases it may be necessary to do a preliminary determination later followed by the final one, where a more suitable concentration of urine is used.

B. Determination of trypsin inhibitor in serum. In this case it proved advantageous to employ a somewhat greater quantity of trypsin, *viz.* 12.5 μg; the period of digestion was reduced to 30 minutes. Serum was diluted with the phosphate buffer solution in the ratio 1 : 200 or 1 : 400, and 1 ml of this diluted solution was used.

Comments on the procedure

It was experimentally found that the combination reaction of the trypsin with the inhibitor of either urine or serum went practically to completion during the fifteen-minute period allowed for this process to take place. The loss in tryptic activity was proportional to the quantity of inhibitor present until about 90 per cent of the trypsin had been inhibited. It turned out, however, that the calibration curves (see below) could only be used within a range corresponding to inhibition of at most two-thirds of the trypsin present. If a greater proportion had been inhibited, the urine had to be further diluted. Within this range there was *a fortiori* proportionality between the loss in activity and the amount of inhibitor.

At the pH value of the reaction mixture, *viz.* 7, trypsin proved so stable that its loss in activity during the fifteen minutes allowed for combination with the inhibitor was negligible.

Exact measurement of the volumes is naturally of great importance. For addition of all liquid volumes of 1 ml or less the use of Carlsberg constriction pipettes is recommended.

Calibration curves

Calibration curves drawn on the basis of the data listed in Table I, which refer to the hydrolysis of the casein substrate by different amounts of trypsin after incubation of the latter for fifteen minutes with 1 ml of the phosphate buffer.

The reference curve for the determination of trypsin inhibitor in urine is shown in Figure 1. Curve a. The shape of the curve suggests that the casein used as substrate contains a small quantity of inhibitor. This is confirmed by Curve b of the same figure, which represents the hydrolysis during an arbitrary reaction time of two hours. This curve intersects the abscissa axis at the same point, in

ble I. *Hydrolysis of casein by trypsin at 30° C.*

determination of rypsin inhibitor n urine (65 min. reaction time)		For determination of trypsin inhibitor in serum (30 min. reaction time)	
rypsin	$E_{280}^{1\ cm}$	μg trypsin	$E_{280}^{1\ cm}$
1	0.047	1	0.018
2	0.130	2.5	0.068
3	0.200	5	0.154
4	0.262	7.5	0.247
5	0.309	10	0.316
6	0.358	12.5	0.378

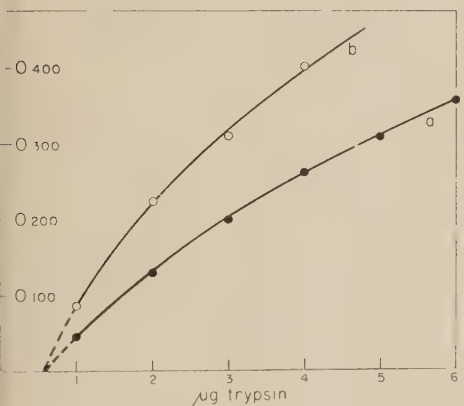

Fig. 1. Splitting of casein by trypsin.

ing that the casein substrate itself inhibits an valent of 0.6 μg trypsin. The part of the curve can be used for the determination of trypsin itor is represented in Figure 2, which may for purpose be regarded as a plot of the difference sorbancies of the standard and of the sample function of the quantity of trypsin inhibited. igure 3 shows the calibration curve used for sin inhibitor determination in serum.

Accuracy

en aliquots of the same urine sample were as- d; the absorbancy observed for any specimen r deviated more than 0.004 from the mean e. This deviation corresponds to about 0.1 μg

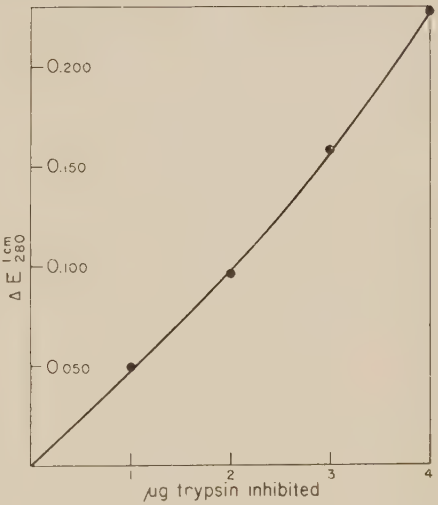

Fig. 2. Reference curve for trypsin inhibitor in urine.

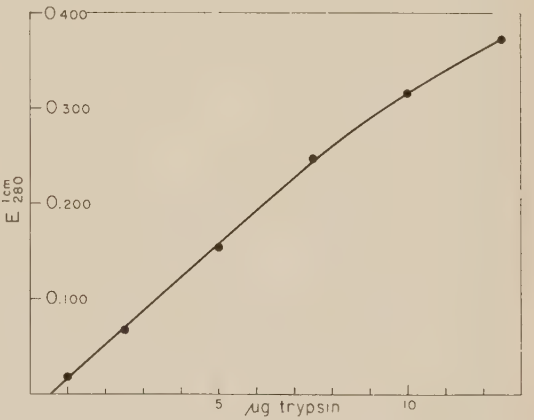

Fig. 3. Reference curve for trypsin inhibitor in serum.

of trypsin inhibited. The experimental volume of most specimens of day-time urine and of pathologic night urine can be chosen so that its content of inhibitor is equivalent to no less than 2 μg of trypsin. Therefore the determination can be carried out to a 5 per cent accuracy or less.

This does not apply to normal night urine which has a very low content of inhibitor. In this case

the experimental error may be much higher than 5 per cent. The excretion of inhibitor in normal night urines varies very much, however, and consequently no greater accuracy is important for its estimation.

If a more exact value is desired, the urine may be previously dialyzed for the elimination of the bulk of the material having a high absorbancy in the ultraviolet. According to Astrup & Sterndorff (1955) the inhibitor found in urine is nondialyzable so that no loss need be feared. After dialysis the urine may be diluted in the ratio 1 : 3.

RESULTS

Dillard (1950) stated that there was a pronounced diurnal variation in urinary excretion of the trypsin inhibitor similar to the variation found for 17-ketosteroids as described by Pincus (1948).

The present author can confirm this observation. Figure 4 refers to an individual experimental subject. During the night the excretion of inhibitor is low, but it rises sharply in the morning. It falls during the first hours of the afternoon, when the subject was reading. It rises again during the afternoon, when he was doing his afternoon ward rounds. After another decrease there is again an increase in the evening, possibly associated with a brief period of laboratory work. Since the rise in the day appears dependent on the physical activity of the subject, the excretion characteristics of a given patient are best evaluated from the night urine, which reflects what might be called the "basal excretion".

In twenty hospitalized patients suffering from indifferent diseases, the excretion varied from 10 to 600 μg of trypsin-inhibiting capacity per hour of diuresis. In hospitalized patients that are kept in bed the diurnal variation is not very pronounced.

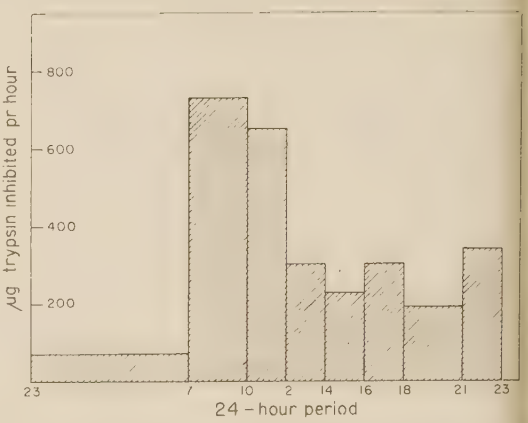

Fig. 4. Diurnal variation in the excretion of trypsin inhibitor in urine.

The author is further able to confirm Dillard's observations (1950) that excretion of the trypsin inhibitor is increased on rise of temperature, after operations, and after other kinds of acute stress. These observations as well as the findings represented in Figure 4 suggest that the excretion of the inhibitor is an unspecific, but sensitive indicator of stress or of muscular activity. A more detailed report will be published later.

As for the content of inhibitor in serum, the author has obtained results in accord with those of Jacobsson (1955). However, the method described here offers advantages over that of Jacobsson.

SUMMARY

A method is described for the quantitative determination of trypsin inhibitors in urine and serum. Since the excretion of the urine inhibitor may be of significance to clinical studies on "stress reactions" the author has made a point of simplicity in the procedure.

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URIC ACID EXCRETION WITH INCREASING PLASMA GLUCOSE CONCENTRATION (PREGNANT AND NON-PREGNANT CASES)

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Literature gives evidence (Talbott, 1943; Bonsnes & Dana, 1946) of some connection between uric acid excretion and plasma glucose. The present paper shows an approximately linear relationship between uric acid excretion and plasma glucose, ranging from a normal fasting level until beyond the level of complete saturation of the renal tubules with glucose. Being interested in the tubular treatment of uric acid during pregnancy, the authors have examined pregnant and non-pregnant women without finding any difference in tubular handling of this compound between the two groups.

MATERIALS AND METHODS

The material consists of 3 normal non-pregnant women and 3 normal women in the 12th, 13th and 16th week of pregnancy. The subjects were hospitalized cases with minor gynecological disorders and without metabolic or renal diseases. All were between 23 and 31 years old.

The clearance determinations were made on fasting persons in the forenoon. Blood samples were taken 2 minutes before the middle of each 10—12 minute clearance period. Urine was collected through an indwelling catheter and after each collection the bladder was washed out twice with 50 ml of sterile water.

The diureses were intentionally heightened, when possible to 5—10 ml per minute, by giving the persons water to drink before and during the first part of the experiment. In spite of this, one subject in each group started off with a diuresis below 2 ml per minute. In most experiments the diuresis was slightly higher at the end of the experimental period than at the beginning.

Inulin clearance was used as a measure of glomerular filtration rate. Plasma levels of inulin were maintained between 20 and 40 mg per 100 ml by continuous intravenous infusion. Inulin was analyzed according to Alving, Rubin & Miller (1939) after yeast fermentation of glucose. Uric acid in plasma and urine was determined by the method of Prætorius & Poulsen (1953). Glucose analyses were carried out according to the method of Somogyi (1945).

Following 3 fasting clearance periods a 20 per cent solution of glucose was infused intravenously and 8—10 clearance periods were made at increasing plasma glucose levels, reaching 400—500 mg per 100 ml.

To make the uric acid excretion per minute directly comparable from subject to subject and from period to period, variations in tubular load have been corrected for by calculating the excretion figures per unity load, E/load, *i. e.* μg excreted per minute/μg filtered per minute, for which the expression Cl_{urate}/Cl_{inulin}, or $R_{u/i}$, is commonly used. The tubular reabsorption is similarly presented as T/load.

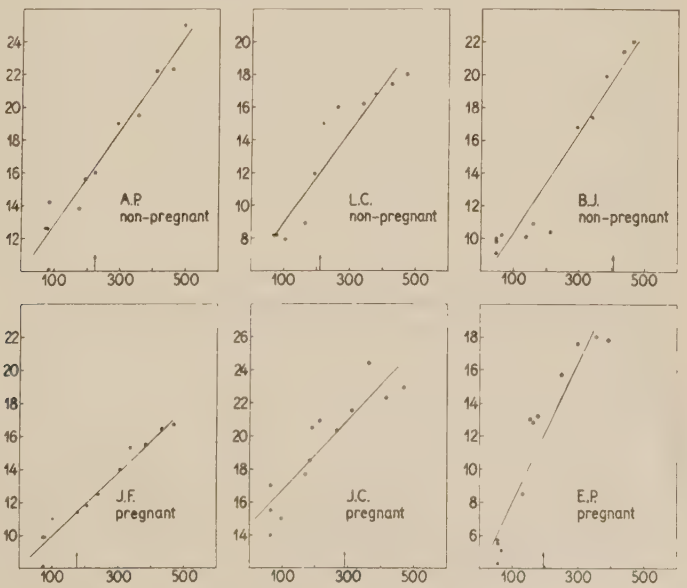
RESULTS

The average glomerular filtration rate was found to be higher in the pregnant group than in the non-pregnant, cf. Table I. The fasting uric acid excretion was correspond-

Table I. *Uric acid, inulin and glucose data from pregnant and non-pregnant women.*

ases		Plasma glucose mg/100 ml	Plasma uric acid mg/100 ml	Diuresis ml/min.	Uric acid excretion mg/min.	Uric acid clearance ml/min.	Inulin clearance ml/min.	$R_{u\,i} \times 100$	T/load × 100
pregnant									
aged 30	fa*	76	4.05	2.1	0.414	10.2	124	8.2	91.8
	hi*	474	3.65	8.8	0.769	21.1	117	18.0	82.0
aged 31	fa	78	1.90	1.9	0.278	14.6	126	11.6	88.4
	hi	490	1.95	7.4	0.750	38.4	154	25.0	75.0
aged 24	fa	48	3.15	6.7	0.313	9.9	103	9.6	90.4
	hi	463	2.70	8.5	0.663	24.6	112	22.0	78.0
ıge	fa	67	3.05	3.6	0.335	11.6 ± 2.6	118 ± 12.7	9.8	90.2
	hi	476	2.75	8.2	0.727	28.0 ± 9.2	128 ± 22.9	21.7	78.3
ıant									
aged 27	fa	56	3.45	4.4	0.246	7.1	138	5.2	94.8
th week)	hi	392	3.05	10.0	0.810	26.6	149	17.8	82.2
aged 24	fa	74	2.70	8.5	0.409	15.1	163	9.3	90.7
th week)	hi	467	2.60	8.0	0.619	23.8	143	16.7	83.3
aged 23	fa	65	2.25	2.9	0.583	25.9	168	15.4	84.6
th week)	hi	468	2.00	8.5	0.664	33.2	145	22.9	77.1
age	fa	65	2.80	5.3	0.413	16.0 ± 9.4	156 ± 16.1	10.0	90.0
	hi	442	2.55	8.8	0.698	27.9 ± 4.8	146 ± 3.1	19.1	80.9

* Fasting state indicated by (fa), high glucose load by (hi). The values at fasting state represent ee collection periods. Uric acid excretion, uric acid clearance and inulin clearance are corrected to 1.73 sq. m. body surface. The deviations given in the table are standard deviations.



1. Ordinate: $R_{u/i} \times 100$. issa: Plasma glucose in mg 100 ml. The lines shown are most probable straight lines gh the dots, calculated ac- ng to the method of least es. The arrows indicate the ıa level at which the renal es are completely saturated glucose.

ingly elevated in the pregnant group, so that the proportion E/load, or $R_{u/i}$, was very nearly the same in the two groups.

During glucose infusion a slight fall in plasma uric acid concentration was seen in 5 of the 6 cases. The average fall appears to be significant ($P < 0.02$). At the same time the uric acid excretion showed a pronounced increase, giving an E/load which rose almost regularly with the increase in plasma glucose concentration, cf. Fig. 1.

There was no noticeable change in the relationship between uric acid excretion and plasma glucose concentration when passing the level of maximal tubular reabsorption of glucose, TmG.

No significant difference in $R_{u/i}$ was found between the pregnant and the non-pregnant group at increasing glucose loads.

DISCUSSION

The elevated glomerular filtration rate found in this investigation in pregnant women (156 ml per minute) as compared to non-pregnant (118 ml per minute) is in agreement with the figures reported by Bucht (1951) and Page, Glendering, Digman & Harper (1954).

The fall in plasma uric acid concentration during glucose infusion may be explained either as due to the enhanced uric acid excretion or to an increase in plasma volume caused by the infusion of hypertonic glucose solution.

The fasting clearance of uric acid found in the non-pregnant group, 11.6 ml per minute, corresponds well with the one reported by Crone & Lassen (1956) using the same method of analysis.

Talbott (1943) has reported an enhanced uric acid excretion following glucose infusion. Bonsnes & Dana (1946) found an elevated uric acid clearance, 2.57 times the fasting values, in 4 normal women in the puerperium, after having elevated the plasma glucose level to about 500 mg per 100 ml. At the same time they measured urea clearance which was found to be increased to a minor degree, 1.25 times the fasting values. The conclusion of their work was that the decrease in rate of uric acid reabsorption occurred only when the tubules were saturated with glucose. This statement is contradicted by the present data which show a roughly linear relationship between uric acid excretion and plasma glucose concentration. The reason for this kind of relationship is difficult to explain.

Urate is most commonly presumed to be freely filtrable through the renal glomeruli and reabsorbed to a degree of about 90 per cent in the tubules. If this be true one might consider a competition between glucose and uric acid to be the explanation of the diminished uric acid reabsorption connected with glucose loading. This, however, seems to be contradicted by the continuance of the linear relationship between E/load ($R_{u/i}$) and plasma glucose concentration far beyond the plasma glucose level at which the renal tubules are completely saturated with glucose at least according to the current conception of TmG.

Poulsen & Prætorius (1954) have forecast the hypothesis of a three component mechanism acting on uric acid in the kidney, the compound being filtered, reabsorbed and secreted simultaneously. If glucose can be thought of as stimulating the secretion of uric acid without influencing its reabsorption

ypothesis might offer an explanation on orrelation between uric acid excretion lasma glucose concentration, but so far s purely speculative.

lersberg, Grisham & Sobotka (1942), son, Levine & Tinsley (1947) and Bene ersley (1951) have reported a non-dif- e plasma urate fraction of 4—20 per A decrease in this fraction possibly d by high concentrations of glucose t have the effect on uric acid excretion ted in this paper. The existence of this usible fraction is, however, denied by ner, Hilton, Yü & Kennedy (1950) and oulsen & Prætorius (1954).

the renal tubular activity is changed g pregnancy towards a series of com- ds, the authors were interested to see her the same was true for uric acid. The nt investigation shows no such differ- in tubular reabsorption of uric acid, r in the fasting state or in the condition ucose loading.

e question of tubular reabsorption of se during pregnancy, only briefly men- d in this paper, will be thoroughly dealt elsewhere (Christensen, 1958).

SUMMARY AND CONCLUSIONS

Uric acid clearance and glomerular tion rate (inulin clearance) have been mined in three normal pregnant and normal non-pregnant women in fasting and with increasing plasma glucose con- ation.

The uric acid clearance is elevated in ancy, but not significantly more than sponding to the increase in glomerular tion.

The uric acid excretion has been found to increase constantly with increasing plasma glucose concentration, almost equally in pregnancy and in the non-pregnant state. The evaluation of this finding is discussed.

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Scandinav. J. Clin. & Lab. Investigation 186—187, 10, 1958.

THE BINDING OF PORPHYRINS TO SERUM PROTEINS

A PAPER-ELECTROPHORETIC STUDY

By T. K. WITH*

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(Received for publication December 4, 1957)

Porphyrins in serum have received scanty attention compared to porphyrins in urine and the serum porphyrins has not been studied with modern methods. As bilirubin, a tetrapyrrole pigment chemically closely related to the porphyrins, is firmly bound to serum albumin when added to serum it is reasonable to assume that the same might be the case with the porphyrins.

MATERIAL AND METHODS

The porphyrins used were pure crystalline methyl esters. Uro- and coproporphyrin I were prepared from bovine porphyria urine as described by With (1958), and uroporphyrin III from turaco feathers (With, 1957), while the preparation of coproporphyrin III was prepared from feces of patients with chronic cutaneous porphyria by Dr. J. Berman, I. Medical Clinic, Karoly University, Praha, Checkoslovakia. The protoporphyrin was prepared from human blood with the method of Grinstein (1947), and the hematoporphyrin was a commercial preparation from Light & Co., England.

The porphyrin esters were hydrolized for 36 hours with 7 N HCl, dried in a vacuum desiccator over P_2O_5 and NaOH after which the residue was dissolved in 2 N NH_3. Solutions containing ca. 1 mg porphyrin per ml were employed of which one drop was added to 1 ml normal human serum, giving a porphyrin concentration of about 5 mg per 100 ml of serum.

The electrophoresis was performed with the apparatus and technic described by With (1956). A control was run with serum to which was added one drop of 2 N ammonia per ml without porphyrins to ascertain whether this addition had any influence on the electrophoretic behavior of the serum proteins. This was found not to be the case. The buffer used was a M/15 barbiturate buffer of pH 8.6, the voltage 400 V, the current 20 mA, the paper Whatman I, and the time 3—4 hours. The movement of the porphyrin was followed during the electrophoresis by radiation with a portable source of Wood's light as described by With (1957). After the electrophoresis the strips were dried, the porphyrins marked with a pencil in Wood's light, and finally the positions of the protein bands were found by staining with bromphenol blue.

RESULTS AND DISCUSSION

All the porphyrins studied, including uroporphyrins I and III, coproporphyrins I and III, protoporphyrin, and hematoporphyrin showed electrophoretic movement completely independent of that of the serum protein bands, the uroporphyrins running much ahead of the albumin while the others followed well behind the gamma globulin.

It was noted that the position of the albumin band was clearly visible in Wood's light as a bluish-white fluorescent zone. Also the other protein bands showed a certain faint fluorescence, but with the lamp employed they could not be clearly distinguished

* Supported by a grant from Frøken P. A. Brandts Legat. With technical assistance of Tove Dreyer Petersen.

is porphyrins added to normal human
n in concertrations of about 5 mg per
ml showed no signs of being protein
l, as is the case with the closely related
bin. Porphyrins are in this respect more
robilinogen which, contrary to bilirubin,
linked to the serum proteins (cf. With,
). We have unfortunately not had acces
ra from cases of porphyria with suffi-
y high porphyrin concentration to make
orphyrin visible after application of the
n to the paper, but as bilirubin added to
al serum is immediately attached to the
nin one would expect the same to be
ase with the porphyrins if they showed
ng to the serum proteins.

SUMMARY

o protein-binding of porphyrins — in-
ng uroporphyrins I and III, copropor-
s I and III, protoporphyrin, and hema-
rphyrin — could be demonstrated by means of paper electrophoresis of normal human serum to which solutions of porphyrin were added to a porphyrin concentration of about 5 mg per 100 ml of serum.

It was noticed that the albumin band was visible as a zone with bluish-white fluorescence in Wood's light on the unstained electrophoretic strip.

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Scandinav. J. Clin. & Lab. Investigation 188—190, 10, 1958.

THE BEHAVIOR OF THE BILIRUBIN-ALBUMIN BOND IN SERUM ON DIAZOTIZATION

A PAPER-ELECTROPHORETIC STUDY

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(Received for publication December 4, 1957)

It is a well established fact that serum bilirubin — "direct" as well as "indirect" — is firmly bound to serum albumin; most recently this has been confirmed in paper electrophoretic studies by Childs (1955) and Klatskin & Bungards (1956). But the fate of the bilirubin-albumin bond when the serum is subjected to the diazo reaction has received only scanty attention.

Bungenberg de Jong (1937) found that azobilirubin formed on diazotization of icteric sera was precipitated with the globulins on half-saturation with ammonium sulphate, but with a very loose attachment, as the azobilirubin was re-dissolved by alcohol. Westphal & Gedigk (1948) found, on study of normal sera with added bilirubin as well as of icteric sera by means of boundary electrophoresis and dialysis before and after diazotization, that the azobilirubin always followed the proteins. But they did not specify whether it was attached to the albumin or the globulins. On dialysis no azobilirubin passed the membrane with direct sera; but if caffeine-sodium benzoate was added as well as the diazo reagent, the azobilirubin passed. Westphal & Gedigk concluded that the bilirubin-albumin linkage remains unchanged during diazotization in direct sera, but this is contradictory to Bungenberg de Jong's findings. We decided therefore to study the question with paper electrophoresis, a simple technic not employed for this purpose before.

MATERIAL AND METHODS

The sera studied were partly from patients with occlusive jaundice with serum bilirubin above 20 mg per 100 ml and partly normal sera to which 1/10 volume of a solution of about 200 mg bilirubin (Hoffman la Roche) in 100 ml N/10 NaOH was added. The reagents and the technic of diazotization were that of Jendrassik & Cleghhorn (1936): 1 volume of serum plus 1/2 volume of diazo mixture being used for the direct reaction, and 1 volume of serum plus 2 volumes of caffeine-sodium benzoate mixture plus 1/2 volume of diazo reagent for the indirect reaction. After the reaction had proceeded for 15 minutes the mixture was applied to the paper.

The electrophoresis was performed as described by With (1956). With sera rich in bilirubin the color of the spots was plainly visible and no staining was required. The buffer was a M/15 barbiturate buffer of pH 8.6, the voltage 400 V, the current 20 mA, and the duration 4 hours. The position of the protein bands was studied after staining with bromphenol blue, but before the staining the position of the bilirubin or azobilirubin was marked with a pencil.

* With technical assistance of Tove Dreyer Petersen.

RESULTS

cteric sera — direct as well as indirect thout addition of diazo reagent the bi- n followed the albumin band very clo- s a narrow band about half as wide as bumin band located in the hind (catho- part of the latter, and this was neither ed by addition of caffeine-sodium ben- reagent nor by addition of diazo re- in the case of indirect sera (normal with added bilirubin). If the amount azo reagent used was insufficient to all the bilirubin to react, the remaining bin also followed the albumin. But azo- bin behaved quite differently, as it was s located more or less behind the a globulin band, sometimes closely be- at other times one to two centimetres d it. Often the azobilirubin band con- of two parts, a blue band in front ic) and a pink one behind, but this on was not constant, the pink band absent in several cases and not visible gh the entire breadth of the strip in . This phenomenon must be due to the nat azobilirubin is an indicator consist- f red and blue components in propor- varying with the pH.

nature and position of the serum pro- ands were not influenced by the addi- f the reagents, with the exception that ecame less intense in proportion to the n.

DISCUSSION

results clearly show that the bilirubin- in bond is opened when the diazo reac- kes place while the mere addition of agents does not influence it. The diazo on takes place at the central methylene bridge of the bilirubin molecule, leaving its other parts intact, and two isomer dipyrrole pigments are formed, the mixture of which constitutes the so-called azobilirubin (Fischer & Haberland, 1935; Yamaoka & Kosaka, 1951; Overbeek, Vink & Deenstra, 1955; Hosokawa, 1956). The bilirubin-albumin bond is believed by some to be located in the two methene bridges on either side of the central methylene bridge of bilirubin, but also the vinyl groups have been mentioned in this connection (Barac, 1946; Kimura, 1953), and finally the phenolic groups may be considered. But neither of these possibilities explain why the bilirubin-albumin bond is dissociated by the diazo reaction which only attacks the bilirubin molecule at the central methylene bridge. The explanation may be that the diazo reagent dissociates the albumin linkage by reacting with the albumin without interfering with the bilirubin outside its central bridge, a not unlikely view as Bungenberg de Jong found that the bulk of the diazonium molecules was bound to the serum proteins and only a minor part was left to react with the bilirubin.

SUMMARY

Paper electrophoretic studies on icteric sera showed that the bilirubin-albumin bond is dissociated during the diazo reaction in both direct and indirect sera. While bilirubin moves in close coincidence with serum albumin, azobilirubin follows behind the gamma globulin without being attached firmly to it. The dissociation of the bond seems not to be due to a reaction with the bilirubin molecule but with the albumin.

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Scand. J. Clin. & Lab. Investigation 191—192, 10, 1958.

A PAPER ELECTROPHORETIC AND PAPER CHROMATOGRAPHIC STUDY OF PENTDYOPENT

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(Received for publication December 17, 1957)

studying bile pigments by spectroscopy, is (1870) described a substance which, treated with alkali and reducing agents, l red and had an absorption band at 1μ. Later the same substance was de- by Bingold (1934), who termed it yopent according to the absorption When hemoglobin, hemin, coproporn and bile pigments, except stercobilinand stercobilin, were exposed to hydroeroxide, a colorless compound termed ntdyopent was formed Bingold (1946). was changed to pentdyopent when d with potassium hydroxide and dithio- Protoporphyrin also gave rise to pentnt formation (Hulst & Grotepass,). The pentdyopent reaction is a group on (Fischer, Haberland & Müller, not obtained with substances containne pyrrole ring, but given by many methenes and oxypyrromethenes. Both and alkaline pentdyopents could be obl from hematin, bile pigments giving o acid ones only (Fischer, 1940). In to obtain further knowledge of these yopents, the use of paper electrophoand paper chromatography was studied e present work.

MATERIALS AND METHODS

Into a 12.5 per cent solution of NH_3 were dissolved hemoglobin (Heyl & Co.) to give a 500 mg per cent solution and hemin (Hoffman La Roche), bilirubin (Hoffman La Roche) and urobilin (Bios Laboratories) to give 20 per cent solutions. Urine from patients with obstructive jaundice and normal human bile in 12.5 per cent solutions of NH_3 were also studied. Propentdyopent was prepared by adding 0.1 ml of a 30 per cent solution of H_2O_2 to five ml of each of the above mentioned solutions and allowing them to remain in a waterbath for four min., after which water was added to volume.

Paper electrophoresis was carried out with barbital buffer, pH 8.6, ionic strength 0.05, using six strips 4 × 34 cm of Munktell 20/100 paper at each run including one strip with glucose to measure the electro-osmotic flow, time 2 hrs. and voltage 425—360 V.

Paper chromatography was performed on Whatman No. 1 filter paper using propanol, NH_3 and water (60 : 30 : 10) as solvent.

The detection on paper occurred as follows: The dried paper was sprayed with a solution containing 5 ml ammonium sulphide and 45 ml of a 10 per cent solution of potassium hydroxide. The paper was dried for 5 min. at 130° C. The propentdyopent was then turned to pentdyopent giving rise to a rather unstable pink color.

RESULTS AND DISCUSSION

In the paper electrophoresis under the above mentioned conditions the propentdyo-

pent obtained from hemoglobin, hemin, bilirubin, urobilin, icteric urine and normal bile, stained as described, moved in two fractions 2.6—3.1 cm and 6.1—6.7 cm towards the anode; in this time the glucose moved 2 cm towards the cathode. By allowing the hydrogen peroxide to act only 1 min. instead of 4 min., only the slower moving fraction is obtained from bilirubin and urobilin. Both the fractions contained acid groups and no alkaline fraction could be observed when using acid buffer systems. In paper chromatography, propentdyopent gave rise to two spots Rf 0.61—0.71 and 0.80—0.85. Solvent systems other than the above mentioned, such as pyridine, almyl alcohol and water (35 : 35 : 30), butanol, acetic acid and water (40 : 10 : 50) and 80 per cent pyridine were tried but with poor results.

SUMMARY

Paper electrophoretic and paper chromatographic technics were applied in the study of pentdyopents. A color reaction for detecting propentdyopent on paper is described. Propentdyopent obtained from hemoglobin, hemin, bilirubin, urobilin, icteric urine an normal bile gave rise to two fractions in bot paper electrophoresis and paper chromato graphy. Both fractions contained acid group as shown in electrophoresis.

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DETERMINATION OF PORPHOBILINOGEN, OPRO- AND UROPORPHYRIN IN URINE AND TISSUE*

A PRELIMINARY REPORT

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(Received for publication December 17, 1957)

ing ampholytes, porphobilinogen, copro- uroporphyrins are present as anions ghtly acid, neutral or alkaline solutions can be retained by anion-exchange re- Methods based on this fact have been in determinations of porphobilinogen urine by Rimington, Krol & Tooth 6) and Mauzerall & Granick (1956). is paper are described the principles of thod which is based on the use of an n-exchange resin for the determination pro- and uroporphyrins, in addition to hobilinogen, from urine and tissue.

MATERIALS

r 2: Cross-linkage 8 per cent, mesh 20—50.

ch's reagent (as used by Mauzerall & Granick, 956): 1 g of p-dimethyl aminobenzaldehyde, ml of 70 per cent solution of $HClO_4$ added to ake 50 ml with glacial acetic acid.

hobilinogen, copro- and uroporphyrins contain- ng urine and tissues: Obtained from acute ex- erimental porphyria in rabbits poisoned with Sedormid, 200 mg/kg and day by stomach tube.

oporphyrin I and III and uroporphyrin I as ethyl esters: A generous gift of Professor . Rimington, F. R. S., Department of Che- nical Pathology, University of London.

orphyrin III: Obtained from acute porphyria rine of rabbits poisoned with Sedormid; iso- ated as methyl ester and purified by chromato- raphy.

Aided by a grant from the Signe and Ane nberg Foundation.

METHODS

Urine, diluted if necessary, or homogenized tissue was added to regenerated Dowex 2 and shaken. The resin was rinsed with water and the porphobilinogen was displaced with a solution of 50 per cent acetic acid. From this solution the porphobilinogen was determined with Ehrlich's reagent in a Beckman B spectrophotometer at wavelength 555 mμ.

The rest of the acetic acid solution was saved for the coproporphyrin determination. The solution was made to react negatively to Congo red paper by the addition of a saturated solution of sodium acetate, the coproporphyrin was extracted with ether, the ether was washed with a 3 per cent solution of sodium acetate and water, any coproporphyrin in the washing water was extracted with ether, and the coproporphyrin was extracted from the combined ether solutions with 0.1 n hydrochloric acid and measured in a Beckman D. U. spectrophotometer at wavelengths 402, 430 and 380 mμ.

The resin was then rinsed free from excess acetic acid with water and allowed to dry at room temperature. The dry resin was then treated with a solution of methanol saturated with hydrogen chloride vapor, by which procedure the uroporphyrin was eluated from the resin. When the solution was allowed to stand in a closed bottle at room temperature for 24 hrs., esterification of the uroporphyrins present took place. After dilution of the solution with water, the porphyrin esters were taken up with chloroform which was allowed to evaporate. Then 7 n hydrochloric acid was added and the uroporphyrin was measured after four days in a Beckman D. U. spectrophotometer at wavelengths 410, 430 and 380 mμ.

RESULTS AND DISCUSSION

In studying the effect of various factors influencing the determination of porphobilinogen it was observed that porphobilinogen from urine was completely retained by the resin. The solution from which porphobilinogen was removed was concentrated under reduced pressure and tested for porphobilinogen by paper chromatography or paper electrophoresis, but no porphobilinogen could be detected. Storage of the porphobilinogen retained by the resin seemed to influence the determinations. Washing of the resin with water should be continued until the water gives a negative test for urea. The strength of the acetic acid solution used for displacing the porphobilinogen was based on determinations made with 100, 50, 25 and 10 per cent solutions of acetic acid containing a constant amount of porphobilinogen. The determinations with a 50 per cent solution of acetic acid gave the highest photometre readings. The amount of acetic acid solution to be used was tested by displacement with an additional amount of acetic acid. The result of porphobilinogen determination from this solution was negative. As porphobilinogen is known to be converted into uroporphyrin in an acid solution, the values will then decrease. However, 4 hrs. seemed to be without greater influence on the results. In testing the ratios of reagent to the solution under determination, maximal values were obtained if the ratio was 2:3. The error of the method applied to urine and tissue was seldom greater than ± 6 per cent, and usually smaller. Determinations carried out with purified porphobilinogen solutions and determinations from tissues to which the same amount of porphobilinogen solution had been added gave a recovery of 80—95 per cent from the tissues.

Concerning the determination of coproporphyrin from urine, it was observed that the coproporphyrin was completely retained by the resin, as was the case with porphobilinogen. The displacement by the 50 per cent solution of acetic acid was nearly complete. When the resin was dried and treated with methanol saturated with hydrogen chloride vapor and the further treatment was continued as for determination of uroporphyrin, a weak line of coproporphyrin ester could sometimes be observed when paper chromatography was carried out with the chloroform solution possibly containing coproporphyrin ester. In testing which isomer of coproporphyrin occurs in the urine of Sedormid-poisoned rabbits, only isomer III was found in paper chromatography of the porphyrin esters (Rappoport, Calvert, Loeffler & Gast, 1955). In determining coproporphyrin from tissues it must be kept in mind that some amount of protoporphyrin is retained by the resin and can be displaced with acetic acid. In the determinations the 0.1 n hydrochloric acid solution is washed with chloroform to remove the protoporphyrin. In acute porphyria, for example, porphyrins other than copro- and uroporphyrins may be present and they may be demonstrated by paper chromatography. The error of the method is within ± 5 per cent for urine containing a large amount of coproporphyrin. For urine containing a smaller amount and for tissues in general, the error is ± 10 per cent. The recovery using pure coproporphyrin III is 90—95 per cent.

Uroporphyrin from urine was also completely retained by the resin. The acetic acid

on which displaces the porphobilinogen he coproporphyrin from the resin causes ht loss of uroporphyrin. The elution of orphyrin from the resin with methanol ated with hydrogen chloride vapor was quite complete. By treating the resin, the above described elution, with hydro- ic acid a weak red fluorescence was ob- d in this hydrochloric acid solution. ving out paper chromatography with the oform solution containing the uropor- n ester and using chloroform and light leum ether as solvent, ratio 1 : 1, and orphyrin I and III as markers there sometimes obtained two fractions with tense red fluorescense moving more y than both the uroporphyrins. The ions were eluated with chloroform, olysed with 7 n hydrochloric acid and ed in paper electrophoresis using uro- nyrin as marker. The fractions moved as as uroporphyrin. The reason for the er movement may have been an incom- esterification. When the uroporphyrin obtained from the urine of Sedormid- ned rabbits is tested by the method of & Benson (1953) it is observed that the er was uroporphyrin III. In the treat- of the resin with methanol saturated hydrogen chloride vapor, some pigments eluated from the resin, and probably an ent substance which made it difficult to lve the uroporphyrin ester in 7 n hydro- ic acid. Dissolving was facilitated by ing the solution, and the pigment factor for the greater part removed by shaking olution with chloroform. To prevent the oration of chloroform, ether or light leum ether was added to the chloroform on and the uroporphyrin ester was ex- d from the mixture with 7 n hydro- chloric acid. However this procedure was rejected because the extraction was incomplete. It was found that uroporphyrin could also be displaced from the resin with hydrochloric acid after the resin was freed with acetic acid from porphobilinogen and coproporphyrin. However, the amount of hydrochloric acid solution required is too large to allow determination of small amounts of uroporphyrin, unless the latter is extracted from the hydrochloric acid solution. This was done, after adjustment of the pH to 1.5 with cyclohexanone according to the method of Dresel, Rimington & Tooth (1956).

The method for determination of uroporphyrin is laborious, but the error when using pure uroporphyrin was only ± 5 per cent and the recovery 70—80 per cent. The same accuracy and recovery were obtained when adding uroporphyrin to tissues *in vitro,* but in the urine and tissue experiments with Sedormid-treated rabbits the error sometimes exceeded ± 10 per cent.

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HIGH-VOLTAGE PAPER ELECTROPHORESIS. A RAPID METHOD FOR DETERMINATION OF URINARY AMINO ACIDS

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(Received for publication December 12, 1957)

The determination of amino acids in urine and blood in association with clinical diagnostics is generally performed with two-dimensional paper chromatography (Dent, 1948; Bickel & Souchon, 1955).

In general, however, when aiming at more accurate quantitative determination, it has been necessary to resort to methods based on ion exchange resins (Moore & Stein, 1951). However, these methods are laborious, and therefore they have not been employed outside scientific laboratories. Electrophoresis has been little used in investigation of amino acids. The reason for this is that separation is poor, due to the rapid diffusion caused by the small molecular size. In order to diminish the disturbing effect of the diffusion, high-voltage electrophoretic apparatuses have been developed, with which it has been possible to reduce the duration of the analysis to a considerable degree. According to this principle and by using apparatus of various types, analysis of peptides, amino acids and the other compounds of residual nitrogen has been carried out with great success (Michl, 1951; Kickhöfen & Westphal, 1952; Heilmayer, Clotten, Sand, Sturm & Lipp, 1954). However, in connection with clinical diagnostics, this method is still infrequently employed (Nöller, Stelgens & Kieser, 1957).

For nearly one year experiments have been carried out at the Children's Clinic in Helsinki according to Werner & Westphal's (1955) method for determination of glutamine in serum, in association with the so-called gliadin tolerance test (Puranen, Puranen & Hallman, 1958). The following is a report of observations on the applicability of high-voltage electrophoresis for determination of urinary amino acids with an immediate view to clinical requirements.

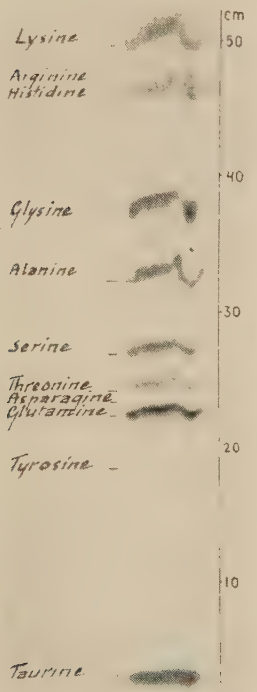
QUALITATIVE ANALYSIS OF AMINO ACIDS

Method

In preliminary treatment of the urine, the samples were filtrated through a membrane made by a dialytic tube for removal of protein, if any, under pressure amounting to approximately 2 kg/cm².

The actual electrophoresis was carried out with an apparatus delivered by the firm of Dr. Virus K. G. Laboratoriumseinrichtungen für Industrie und Wissenschaft, Bonn.

As buffer solution 0.75-N formic acid and 1-N acetic acid mixture (1 : 1) (Gross, 1956) was used. The pH-value of the buffer was 2.25. On a slip of electrophoretic paper (S & S 2043 b), 15 cm in width, the sample of urine (10—100 microl) was pipetted in the form of a band 3 cm in length being simultaneously subjected to dry ice refrigeration in order to prevent spreading. Two samples



1. High-voltage electropherogram of urine
ted by a healthy child. 30 microl urine con-
g 500 microg total nitrogen and 7.5 microg
amino nitrogen was pipetted on paper.

he required standard could thus be pipetted on
aper. The strength of the current employed
1.0—1.1 mA/width in cm, and the voltage 55
gth in cm. The temperature of the refrigerat-
quid was —13—15° C and the duration of the
sis 150 min. The paper was subjected to dry-
or 7 min. at a temperature of 80° C. To make
np, the paper was sprayed with a ninhydrin
on (0.1 per cent ninhydrin in ethanol con-
g 5 vol per cent 2, 4, 6-collidine) (Block *et al.*,
. The color was developed by keeping the
for 10 min. at temperature of 80° C.

Comments on the method

eliminary treatment of the urine: Re-
l of proteins, if any. Removal of non-organic salts, on the other hand, is not required, since they hardly affect the intensity of the color in the pherogram at all. In pure state, a given amount of standard amino acid at testing displayed an equally high and clear color as when added to urine, although there was a difference in the migration of the samples.

Choice of buffer: By testing various buffers it was established that the buffer actually employed was liable to give the most distinct result (Puranen, J., personal communication). The following theoretical arguments, moreover, influenced the choice of buffer: 1) All amino acids are at this pH positively charged, and consequently migrate in the same direction, 2) the ionic concentration is sufficient for the current employed, 3) on drying the buffer will entirely disappear from the paper.

Operating voltage and duration: The voltage limit tolerated by the refrigeration effect was used. The duration was empirically chosen with due consideration to the fact that, on lengthening in time, the reciprocal differences of the various amino acids become greater, whereas the results will be more indistinct because of the spreading.

Speed of migration of the various amino acids: Table I presents the relative migrations of the amino acids in electrophoresis in comparison with the distance covered by glutamine (R_{glut}) which is indicated with numeral 1. The average speed of glutamine was 1.71 mm/min. Separation of most of the amino acids present in the urine was good. Identification of glutamine, glutamic acid and methionine, however, was impossible. Proline was identified because of its color in the lower margin of the spot produced by

Table I. *The relative migrations of the amino acids determined by adding pure preparations into the samples of urine.*

Amino acid	$R_{glut.}$
Taurine	0.14
Fosfoethanolamine	0.19
Tryptophan	0.70
Oxi-proline	0.78
Tyrosine	0.83
Aspartic acid	0.88
Cystine	0.89
Phenylalanine	0.93
Proline	0.95
Glutamic acid	0.97
Glutamine	1.00
Methionine	1.02
Citrulline	1.02
Asparagine	1.04
Threonine	1.08
Leucine	1.12
Iso-leucine	1.15
Valine	1.18
Serine	1.21
Alanine	1.44
Glycine	1.69
β-amino-iso-butyric acid	2.04
Histidine	2.06
1-methylhistidine	2.06
Arginine	2.10
γ-amino-butyric-acid	2.16
Lysine	2.25
Ornithine	2.30
Ethanolamine	2.90

this group. 1-methylhistidine and histidine could not be separated from each other. It was difficult to separate cystine and aspartic acid as well as leucine and iso-leucine. The migrations of the amino acids were nearly constant, with the exception of taurine which displayed considerable deviation. Table II presents the relative migrations of six common amino acids and the corresponding standard deviations on the basis of ten different electropherograms.

Identification of the amino acids: Table III gives an illustrative example of the qualitative results of electrophoresis carried out on unequal amounts of the same sample of urine.

Table II. *The standard deviation of the relative migrations (R glut) of six common amino acid and the corresponding percentage determined on the basis of ten different electropherogramms.*

Amino acid	$R_{glut.}$ average	Standard deviation	Standard deviation percentage
Taurine	0.14	±0.023	16.0
Threonine	1.08	±0.019	1.8
Serine	1.21	±0.010	1.2
Alanine	1.44	0.053	2.7
Glycine	1.69	0.028	1.7
Histidine	2.06	0.035	1.7

The identification of the amino acids is performed by adding pure preparations into the samples and by following their migration in electrophoresis. The hydrolytic resistance of the identifiable colored spots was established by hydrolysis.

The identification of ethanolamine cannot be considered positive, and the same colored spot may also contain other amines, judging by its disproportionately intense color.

QUANTITATIVE DETERMINATION OF AMINO ACIDS

In addition to the qualitative analysis we attempted to develop a method for quantitative determination of the amino acids present in ample measure in the urine. Determination of amino acids occurring in all electropherograms proved impossible within the range of one analysis.

Table III. *The qualitative results of analysis carried out on unequal volumes of same urine sample. The sample was urine of a 3-years old healthy boy. Key to the signs: + amino acid clearly identifiable. ± vaguely identifiable.*

ne	+	+	±									
ie	+	+	±	±	±							
ine	+	+	+	±	±							
ylalanine ..	+	+	+	±	±							
sine	+	+	+	+	±							
ie	+	+	+	+	+	+	+	±				
iine	+	+	+	+	+	+	+	±				
nolamine(?)	+	+	+	+	+	+	+	+				
ragine	+	+	+	+	+	+	+	+	+			
nine	+	+	+	+	+	+	+	+	+	±	±	
line	+	+	+	+	+	+	+	+	+	±	±	
ne	+	+	+	+	+	+	+	+	+	+	+	+
ne	+	+	+	+	+	+	+	+	+	+	+	+
e	+	+	+	+	+	+	+	+	+	+	+	+
mine	+	+	+	+	+	+	+	+	+	+	+	+
ine	+	+	+	+	+	+	+	+	+	+	+	+
ne mikrol..	110	100	90	80	70	60	50	40	30	20	10	5
o nitrogen rog	18.3	16.6	14.8	13.2	11.5	10.0	8.3	6.6	5.0	3.3	1.7	0.8
nitrogen rog	791	716	644	572	501	429	358	286	215	143	75	36

Procedure

ter the electropherogram was stained, the ed spots produced by the amino acids subjected alysis were cut off and their superficial areas measured with a planimeter. Each colored was introduced into 4 ml solution containing $CuSO_4 \cdot 5\ H_2O$ in 100 g 75 per cent ethanol k *et al.*, 1955). The color was measured after apse of half an hour with the Beckman rophotometer, at a wave length of 520 mμ histidine 400 mμ). From the clean area of the ophoretic paper, two to three unequal strips cut off, the superficial area and color of were measured in the same way as those of amples. With the aid of these measurements s possible to determine the color value of the paper" to be deducted from the color value e amino acid to be measured. In association each analysis the color value of the given t of standard amino acids was determined. andard, 5 microg glutamine was used. The color values of the amino acids to be determined were then compared with the standard, and the results were stated as glutamine units (GU). The amount of amino acid is 1 GU when it gives an equally intense color as 1 microg glutamine under similar conditions. Furthermore, by using pure preparations, the absolute amounts of the amino acids — in a way they make a ratio of the ninhydrinic sensitivity — corresponding to the glutamine unit, were empirically determinable.

These coefficients were: 1 GU = 3.6 microg histidine, 0.91 serine, 0.8 threonine, 1.02 taurine, 0.43 alanine and 0.82 glycine.

Results

Table IV presents the results of seven determinations worked out in parallel on the same sample, as well as the standard deviations. The percentage of error increases with longer migration and amino acids tending to

Table IV. *Quantitative parallel determination. Results of seven determinations worked out on the same sample. 60 microl urine was used for each determination corresponding to 500 microg total nitrogen and 9.1 microg amino nitrogen.*

Amino acid	Glutamine-units in various determinations							Average	Standard deviations	Standard deviations in per cent
	1	2	3	4	5	6	7			
Taurine	2.5	4.1	4.5	5.8	2.8	1.8	2.6	3.5	1.3	37.0
Glutamine	5.6	5.6	6.2	5.5	6.3	6.0	5.5	5.8	0.3	5.2
Serine	3.2	3.1	3.4	4.8	4.5	2.1	3.4	3.5	0.9	26.0
Alanine	5.7	5.8	5.7	6.0	5.5	4.5	5.8	5.6	0.5	9.0
Glycine	5.7	5.5	5.5	4.3	4.7	6.2	4.2	5.2	0.7	13.0
Histidine	1.4	5.5	4.7	4.8	2.5	3.7	4.7	3.9	1.4	35.0

Table V. *Results of electrophoresis carried out for estimation of the standard coefficients of alanine and glutamine.*

Amino acid	Amount microg	Area of spot cm^2	Extinction value 0.00..	Paper reduction 0.00..	Difference 0.00..
Alanine	0.5	2.8	45	30	15
	1.0	4.0	63	33	30
	1.5	5.6	82	38	44
	2.0	5.6	98	38	80
Glutamine	1.25	1.6	37	23	14
	2.50	4.0	66	34	32
	5.0	4.4	100	35	65
	10.0	8.0	174	44	130

spread over an extensive area. The percentage of error for taurine is also high, because this acid, too, tends to spread; nor does it form any distinctly outlined spot in the same fashion as glutamine, for example. The relatively high color value of the paper itself is obviously one of the most important factors in causing the error. For some unknown reason the color value of the paper did not increase linearly, so the standard values of the paper reduction had to be determined for each analysis. Knowing the size of the colored spots produced by the amino acids, we obtained from the standard paper curve the approximately correct "paper reduction".

By determining the standard curves of various amino acids for determination of the ratio of the glutamine unit, and, proceeding as described above, correcting for the paper reduction, linear results were obtained with great accuracy. Table V presents, as an illustrative example, the results obtained on determination of the standard values of glutamine and alanine. The significance of the paper reduction is clearly apparent.

In Table VI we present the result of the 24-hour secretion of amino acids in ten healthy children of different ages. Furthermore, the total amount of amino nitrogen was determined according to the method based on Folin's naphthoquinone reaction (Cagan, Goldberg & Loewe, 1951). The correlation between the results of electrophoresis and the determination of amino nitrogen is fairly good. The results correspond to the results arrived at by other investigators.

able VI. *The excretion of amino acids per 24 hours of ten healthy children. volume of urine was used, the total nitrogen of which amounted to 500 microg. electrophoretic analysis the amounts of seven frequently occurring amino acids were determined.)*

Months	Total nitrogen g/24 hrs.	Amino nitrogen mg/24 hrs.	Taurine mg/24 hrs.	Glutamine mg/24 hrs.	Threonine mg/24 hrs.	Serine mg/24 hrs.	Alanine mg/24 hrs.	Glycine mg/24 hrs.	Histidine mg/24 hrs.	Total mg/24 hrs.
2	0.6	40	12	12	-	12	6	17	6	65
4	4.3	99	37	44	-	21	14	30	18	164
2	2.8	80	28	28	-	11	10	18	37	132
1	6.2	156	140	55	19	24	14	27	110	389
8	3.1	58	9	19	-	10	9	12	58	117
1	5.8	91	81	31	-	8	7	6	40	173
2	7.5	140	103	55	-	16	6	31	20	231
8	3.9	75	33	31	-	17	5	20	27	133
9	5.5	95	17	53	-	28	16	61	88	260
8	13.0	232	200	90	-	32	25	39	90	476

DISCUSSION

igh-voltage electrophoresis is an exceed- rapid and simple method for one ed in the technics for determination of ary amino acids. In general, separation od. Even quite insignificant quantities nino acids become visible by this method. ntitative estimation can be done either by l means, or, for example, in accordance the method presented. By developing nethod, particularly with the quantitative result in view, accuracy may be positively eased. One of the drawbacks of the me- is the high price of the required appa- s as well as the reciprocally poor identi- ion of some amino acids when employ- ne buffer.

SUMMARY

bservations on the applicability of high- ge electrophoresis for determination of ary amino acids are presented. The od is rapid and separation of amino acids good, excluding a few exceptional cases. Results of semiquantitative determinations are presented.

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MICRO-METHOD FOR DETERMINATION OF BLOOD GALACTOSE BY MEANS OF GLUCOSE OXIDASE (NOTATIN) AND ANTHRONE

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(Received for publication January 4, 1958)

antitative determination of the urinary tion of galactose after administration of tose by mouth has been used to some t as a liver-function test since Bauer ibed his galactose-tolerance test in 1906. wever, determination of the blood-galac- concentration after ingestion of galactose more accurate information on the in- y of galactose metabolism. Even though methods are available by which the galactose can be determined by means e of the usual reduction techniques after g removed the glucose by fermentation yeast, this valuable function test has not widely used as a diagnostic aid in cli- routine work. This is, in particular, due e fact that fermentation with yeast is complicated, and requires a very care- chnique if unreliable results are to be ed.

gstrup, Winkler, Lund & Engell 4), in their liver-function tests, used se oxidase (notatin) instead of yeast in etermination of the galactose concentra- which must be regarded as a substantial ovement.

galactose-tolerance tests in infants — for example, if galactosamia is suspected — it is necessary to use a micro-method for the analysis of capillary blood. In a case of galactosemia reported by Mortensen & Søndergaard (1954, 1955), fermentation with yeast was used in combination with titration by the method of Hagedorn and Norman Jensen. With a view to another case of galactosemia encountered by the same authors (report in preparation), I elaborated in 1955 a micro-method in which glucose oxidase and anthrone are used. This method, with a few minor modifications, is decribed in detail below.

METHODS

Reagents

The following reagents were used:

A 3.5 per cent solution of primary potassium phosphate (KH_2PO_4). A fresh solution was prepared at intervals of a few days and stored in a refrigerator.

Glucose oxidase (Notatin LEO [2]) was stored at —18° C (stable for several months in the deep-frozen state). A fresh solution containing about 1 mg glucose oxidase in 1 ml of 3.5 per cent primary potassium phosphate was prepared daily.

Barium hydroxide, 0.3 N (4.73 per cent Ba $(OH)_2$, 8 H_2O).

ided by a grant from the King Christian the Foundation. Present adress: Amtssygehuset, rg, Denmark.

[2] Notatin LEO for experimental use was generously supplied by Leo Pharmaceutical Products, Copenhagen.

Zinc sulphate, 10 per cent ($ZnSO_4$, 7 H_2O).

Anthrone reagent, *viz.* 1 g Anthrone (Hopkin & Williams) dissolved in 500 ml dilute sulphuric acid diluted by adding in small amounts 1000 ml of concentrated sulphuric acid for analysis to 200 ml of water (Riedel-de Haën)). Stored in a refrigerator, the anthrone solution is stable for 1—2 weeks.

Galactose standard, *viz.* 100 mg/100 ml solution of pure galactose (Keerfoot). Stored in a refrigerator, the solution is stable for at most 1 week.

Freshly redistilled water.

Procedure

To 1 ml of water pipetted into a small test tube (100 × 14 mm) is added 0.1 ml of blood. After shaking, the tube is allowed to stand for about 2 minutes until hemolysis has occurred.

By means of a Carlsberg pipette 0.2 ml of the solution of glucose oxidase in phosphate is then added. It is important that any drops of the blood dilution on the wall of the test tube be mixed with the glucose oxidase solution. The tube is closed with a rubber stopper and placed in a water bath at 37° C for 90 minutes, the samples being shaken very thoroughly at intervals of 20—30 minutes.

Next, 0.5 ml of barium hydroxide is added. After mixing and standing for 3—4 minutes, 0.2 ml zinc sulphate is added. After renewed mixing and standing for about 2 minutes the sample is centrifuged for 30 minutes at 3 000 r. p. m. However, a better procedure is to centrifuge for 10 minutes and then recentrifuge the supernatant fluid for another 10 minutes.

By means of a Krogh syringe pipette (the procedure is facilitated by using a type with two adjustable stops), 5 ml of the anthrone-sulphuric-acid reagent is delivered into a Pyrex test tube, which is then cooled in ice water. By a Carlsberg pipette, 1 ml of the clear supernatant fluid is transferred from the centrifuge tubes to the anthrone tubes in such a manner that the fluid is allowed to run along the wall of the tube, forming a layer on the heavy sulphuric acid. The two substances are carefully mixed under constant cooling, so that a rise in temperature is avoided. The tubes are closed with Kapsenberg capsules. By means of a wire basket, the tubes are placed in a vigorously boiling water bath after removal of any lumps of ice adhering to the tubes. In order to obtain uniform heating the water in the boiler must reach about 2 cm above the fluid surface of the test tubes and a grid be placed on the bottom of the boiler. The heating is interrupted after exactly 4 minutes. The samples are at once placed in ice water and afterwards at room temperature (direct sunlight should be avoided). The reaction may be read at once, but if the reading is postponed, the samples should be placed in a refrigerator. The reading is performed in a spectrophotometer (Zeiss "Spepho") at a wavelength of 620 mμ.

The galactose standard and blank are determined in the same way, the only difference being that 0.1 ml galactose standard and 0.1 ml water, respectively, are used instead of blood.

If it is desired to determine the glucose concentration at the same time, a special set of blood samples is required for the determination of the total sugar extinction. The calculations are facilitated by treating these samples in the same way as the galactose samples, 0.2 ml of phosphate solution being used instead of glucose oxidase solution. The glucose standard and the corresponding blank are determined at the same time.

DISCUSSION

Fermentation

Glucose oxidase is an enzyme that oxidizes glucose to gluconic acid as follows:

$$C_6H_{12}O_6 + O_2 + H_2O \rightarrow C_6H_{12}O_7 + H_2O$$

It was first prepared from *Aspergillus niger* by Ditlev Müller in 1928, and later isolated from *Penicillium notatum* by Coulthard *et al.* (1945), who named it Notatin. In several papers, Keilin & Hartre (1948) reported detailed investigations into the physical and chemical properties of the enzyme and its biological activity. They utilized the oxygen uptake during the enzymatic process in a very accurate and specific method for quantitative glucose determinations in the Barcroft manometer. This method was very useful in the study of biological material;

l be used for glucose determinations in
na, but not in blood because the conver-
of oxyhemoglobin into methemoglobin
ences the oxygen uptake.
eilin & Hartree (1952) found that the
me was highly specific. In studying a
number of carbohydrates, they demon-
ed that in addition to glucose only a few
ese were oxidized, and only to such a
t extent that it was without significance
uantitative glucose determinations. Thus,
rified galactose preparation was oxidized
rate corresponding to 0.14 per cent of
of the oxidation of glucose. Owing to
amination with glucose, all "pure" com-
cial galactose preparations are oxidized
higher degree (up to 4 per cent). In one
tin preparation, the specificity was so
ounced that only beta glucose was oxi-
d, while alpha glucose was oxidized only
tep with the mutarotation. Other pre-
tions also contained an enzyme which
lyzed the mutarotation. The optimum
level for the oxidase was 5.6, but varia-
s within the range from 5 to 6 did not
ence the activity. Phosphate, usually
M, was normally used as a buffer, but
reciable variations in the buffer concen-
on were without influence on the activity.
optimum temperature was found to be
o 40° C, but already at 30° the activity
almost reached its maximum. The pro-
proceeds more rapidly in pure oxygen
in atmospheric air. The oxidase activity
nly to a very slight extent subject to an
bitory influence from other substances.
the method described here a phosphate
centration of about 0.25 M was used in
r to facilitate the protein precipitation.
pH was 5.8 and did not undergo appreci-
able changes during the process. For practical reasons, the samples were incubated at 37° C, because a water bath of that temperature is available in most hospital laboratories. A shaking device such as that used by Tygstrup *et al.* (1954) is obviously very useful, but repeated shaking of the samples by hand is sufficient to ensure the oxygen supply required for the micro-determinations. As appears from Figure 1 and Table I, an in-

T a b l e I. *Sugar concentrations in blood and in glucose and galactose solutions after treatment with glucose oxidase for various periods*

	Glucose oxidase treatment, in minutes			
	0	60	90	120
Blood sugar (mg/100 ml)	102	8	7.5	7.5
Do. (diabetic patient)	305	13	10	11
Glucose solution (mg/100 ml)	200	5	0	-
Do.	500	33	9	-
Do. (notatin in double concentration)	500	12	6	-
Galactose solution (mg/100 ml)	200	200	201	202

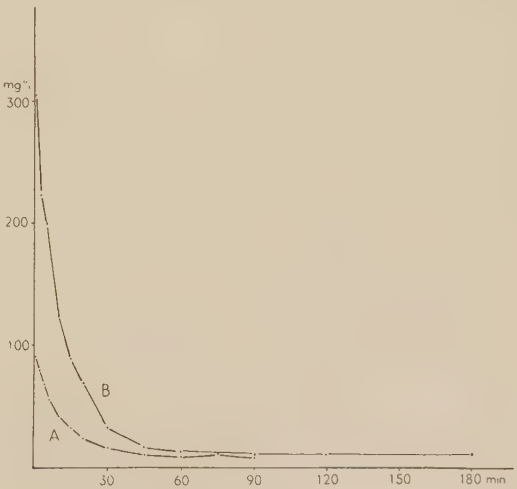


Fig. 1. Variations in the blood-sugar concentration with time during treatment with glucose oxidase. A. Normal blood sugar. — B. Hyperglycemia (diabetic patient).

cubation period of 90 minutes is sufficient, also in the presence of elevated blood-glucose values. Only at excessively increased glucose levels in the blood may it be necessary to extend the incubation time or to increase the glucose oxidase concentration. The anticoagulants in common use, such as oxalate, citrate, complexon and heparin, are without influence on the oxidase activity.

Stored at ordinary refrigerator temperature, notatin is stable for several months; if its stability is to be preserved for still longer periods, it should be stored in a deep-freezer.

Protein Precipitation

The technique employed in protein precipitation is a modification of the method which Somogyi (1945) used for blood-sugar determinations, and which was adapted for photometric use by Nelson (1944).

Somogyi used 0.2 ml of 0.2 N $Ba(OH)_2$ and 0.2 ml of 5 per cent $ZnSO_4$ to 0.1 ml of blood. He found that the protein precipitation was very effective, even if boiling was omitted, and that the precipitated barium sulphate had a high capacity of absorption.

In addition, the method offers the advantage that the total volume can be kept relatively small, which is of importance in determining low galactose concentrations, since galactose gives a somewhat fainter color reaction with anthrone than other hexoses. In order to have as small a volume as possible, a phosphate solution of only 3.5 per cent is used for the oxidase solution. In this way, the amount of barium hydroxide required for the precipitation of phosphate and protein is only 0.5 ml. It is important to ensure complete precipitation of the barium, as it will otherwise be precipitated by the anthrone-sulphuric-acid reagent. Complete precipitation is obtained with 0.2 ml of 10 per cent zinc sulphate. Thus, the total volume becomes 2 ml. It is inappropriate to use smaller volumes, as this may make it difficultl to suck up the amount of fluid (1 ml) which is to be used for the color reaction.

Blood-sugar determinations by the anthrone method using either the Somogyi precipitation or the present modification thereof showed good agreement as appears from Table II.

Table II. *Blood-sugar content in mg per cent in 5 samples as determined by the anthrone method, using the Somogyi precipitation and the authors modification respectively.*

Blood samples	I	II	III	IV	V
Somogyi's method	121	90	104	92	16
Author's modification	122	91	102	91	16

In measuring the amount of barium hydroxide (in which a Carlsberg pipette is also very useful), some barium carbonate may precipitate inside the pipette, which must therefore be changed fairly often. After use the pipettes are easily cleaned in 10 per cent hydrochloric acid.

After the protein precipitation, some particles, which cannot be completely removed by centrifugation, may lodge on the fluid surface. It may be difficult to avoid that these particles are sucked up into the pipette. This difficulty may be overcome by centrifugation for 10 minutes; the supernatant fluid is then cautiously poured into another centrifuge tube and recentrifuged for 10 minutes.

uantitative determination of galactose

ter having tested various techniques for titative sugar determinations, the an- he method was chosen. This substance is anthraquinone derivative. Dreywood 6) showed that it could be used for the nstration of carbohydrate material, be- anthrone dissolved in sulphuric acid on ng with carbohydrate gives a green . The color is presumably due to a re- n between anthrone and the furfural ounds which are formed when carbo- ate is heated in concentrated sulphuric (Sattler, 1948).

ne green color has proved to be highly fic for carbohydrate and carbohydrate- aining material (Dreywood, 1946; Satt- 1948; Morris, 1948; Bonting, 1954, and rs). Apart from carbohydrate, this color ion has been demonstrated only with ural. On the other hand, owing to a re- n with tryptophan, a red color develops ddition of a few substances, including ein.

orris (1948) demonstrated that the re- n could be utilized for quantitative sugar rminations by reading the color intensity photometer at the absorption maximum, h is usually stated to be 620—630 mμ. his wavelength, the red color which may lop due to the presence of other sub- ces is of no significance.

n the other hand, Shetlar (1952) showed tryptophan reacts with sugar and there- brings about a reduction in the color sity of the anthrone reaction, depending he tryptophan concentration. However, ppears from the investigations reported Björnesjö (1955), a relatively high tryp- tophan concentration is required before the color is appreciably affected. At the usual blood concentration of tryptophan, this source of error can be disregarded, especially when precipitation with barium hydroxide and zinc sulphate is used, as this precipitation removes some tryptophan from the blood.

Durham (1950) used anthrone for blood-sugar determinations, a method which may, with advantage, be employed as a rapid screening test (Fetz, 1950).

For the reaction, most authors have utilized the spontaneous development of heat which occurs on addition of the anthrone-sulphuric-acid reagent to the aqueous sugar solution. However, as the resultant color intensity, to a great extent, depends on the temperature of the mixture of the reagents, it may be difficult to obtain completely satisfactory results by this method.

Better results are obtained when the development of heat is regulated. Trevelyan & Hartree (1952) did this by dissolving anthrone in slightly diluted sulphuric acid. To a measured amount of this reagent they added, while cooling with ice, the sugar solution, which formed a layer on the heavy sulphuric acid. After mixing, they at once cooled with ice water, thus avoiding a rise in temperature. The mixture was then boiled for 10 minutes and again cooled in ice water.

Owing to the greater degree of certainty, the procedure described by Trevelyan & Hartree was used in the present method. The only modification is that a slightly less dilute sulphuric acid was used, because it appeared that a certain anthrone preparation did not readily dissolve when the acid was further diluted. The various commercial anthrone

preparations also differed in other respects; thus, one proved useless because of a very considerable blank value. Various qualities of sulphuric acid for analysis may also, although to a less degree, give rise to variations in the blanks.

When stored at room temperature, the yellow color of the anthrone-sulphuric-acid reagent will soon increase in intensity. Stored in a refrigerator, the reagent proved to be more stable; after storage for a fortnight, the blank value had increased so little that it was without significance in the sugar determination; nor was the sugar-anthrone reaction appreciably influenced. However, during the elaboration of the method, a fresh reagent was usually prepared daily.

As appears from Figure 2, the color intensity depends, to a great extent, on the heating time; the color intensity increases fairly rapidly and then again slowly decreases on continued heating. Both the color intensity and the time required to attain its maximum vary with the type of sugar used; thus, the maxima for galactose and glucose were attained after boiling for 3½—4 and 5½—6 minutes, respectively. Accordingly, boiling for 4 minutes should then preferably be used in galactose determinations.

Good proportionality exists for pure galactose solutions in the concentrations which may be expected in blood (Fig. 3), and this proportionality is preserved after treatment with the reagents used for precipitation (Fig. 4).

On storage in darkness at room temperature, the color remains unchanged for at least 4 hours. When stored in a refrigerator, the samples may, if necessary, stand overnight without any harmful effects. On the other hand, in daylight the color begins to fade after ½—1 hour.

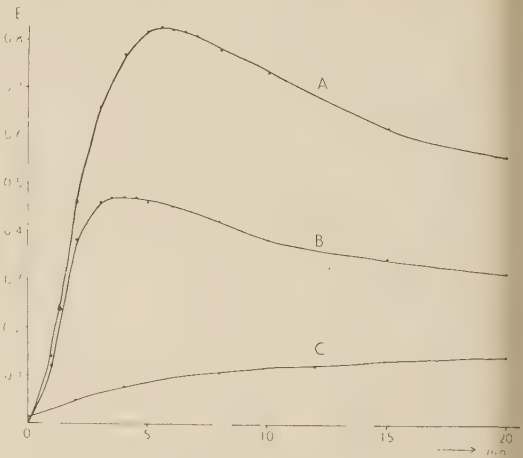


Fig. 2. The dependence of the anthrone-sugar re action on the heating time. A. Glucose solutio 100 mg/100 ml. — B. Galactose solution, 100 mg 100 ml. — C. Blank multiplied by 10.

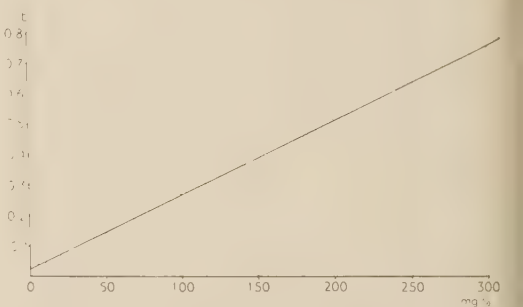


Fig. 3. Optical density for the anthrone-galactose reaction.

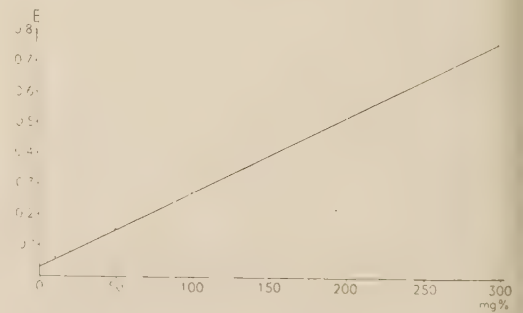
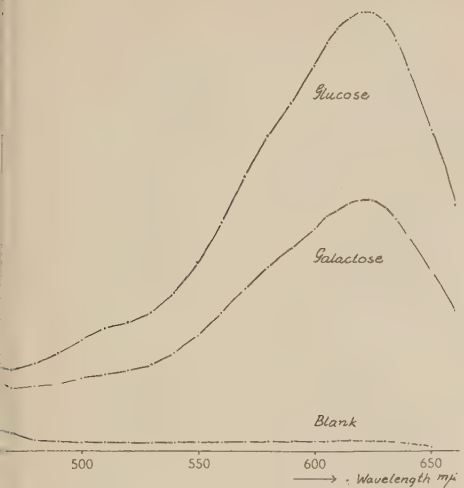


Fig. 4. Optical density for galactose after treatme with the precipitation reagent.



g. 5. Optical density for the anthrone-sugar reaction.

igure 5 shows the curves for the optical sity of galactose and glucose.

n using the anthrone method, it is im- tant that the tubes are carefully cleaned, dust and fluff must be avoided while the ples are pipetted off into the anthrone es, as otherwise, due to the hydrolytic ef- of sulphuric acid, carbohydrates may be rated, which may give rise to consider- errors. Accordingly, the technique can- be recommended as a routine method in inary hospital laboratories.

Applicability of the method in blood-galactose determinations

n order to check the reliability of the hod in determining the blood concentra- of galactose, amounts of 14.4 and 32.6 of desiccated galactose were placed in graduated flasks, which were then filled heparinized blood up to the 10-ml mark. er dissolution and careful mixing, samples

Table III. *Determination of galactose added to venous blood in mg per cent. a. Blood without added galactose. b. Blood with added galactose 144 mg per cent. c. Blood with added galactose 326 mg per cent (after subtraction of the average of 1.)*

	Results of six determinations						Average	
a.	4.0	5.8	4.9	4.9	5.8	5.8	5.2	"gal."
b.	144.7	144.2	147.7	144.7	146.8	147.3	145.9	gal.
c.	320.4	331.1	326.8	329.0	329.0	328.1	327.4	»

of 0.1 ml of blood, which thus had known galactose concentrations of 144 or 326 mg/100 ml, were treated with glucose oxidase in the usual manner. The results appearing from Table III show that the added amounts of galactose could be determined with sufficient accuracy.

Following notatin treatment, venous blood withdrawn from a fasting subject showed sugar concentrations corresponding to 4.9, 3.5, 3.5, 5.3, 3.1, 4.9, 4.4 and 7.1 mg/100 ml galactose, and 1 hour after oral administration of 40 g galactose the values were 43.4, 47.3, 46.9, 46.9, 47.8, 45.1, 45.1, 47.8, 49.2 and 44.2 mg/100 ml.

In another subject, the following values were obtained 15 minutes after intravenous injection of 0.5 galactose/kg body weight: — 156.7, 157.1, 157.1, 157.5, 160.5, 160.1, 161.4, 160.4, 161.4 and 155.8 mg/100 ml.

On notatin treatment of blood, some "residual sugar" remains — in most cases corresponding to 6—12 mg/100 ml galactose (or converted into glucose, 4—8 mg/100 ml). Probably, it consists of monohexoses other than glucose, perhaps polysaccharides of low molecular weight which have not been bound by the protein precipitation. It may also be conceived that it is alpha glucose which has

not been converted into beta glucose by the mutarotation during the incubation period. However, extension of the incubation time resulted in only very small decreases, or none at all, in the sugar concentration; hence, it is unlikely that this plays any significant rôle. Nor did an increase in the notatin concentration result in a reduction in "residual sugar".

SUMMARY

A micro-method for quantitative determination of blood galactose is described. The following reagents are used: glucose oxidase (notatin) for fermentation of glucose, barium sulphate for protein precipitation, and anthrone for photometric quantitative determination of galactose.

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DETERMINATION OF PROTEIN-BOUND RADIOACTIVE IODINE

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(Received for publication January 21, 1958)

is possible to measure the rate at which hyroid gland releases its hormone into lood. Such measurements are often of value in the clinical diagnosis of thyroid rders. The procedure consists in deter- tion of the protein-bound radioactive e (PBI^{131}) in serum after a specified interval following ingestion of a tracer of NaI^{131}.

the standard procedure, serum protein ecipitated by trichloracetic acid or an- r protein precipitant. The precipitate is ed repeatedly, and while it is still wet, adioactivity is measured by means of a illation counter.

though the method is so simple, its ac- cy is still open to doubt. The general ion is that values in the normal range ot be determined to a satisfactory ac- cy (Mac Gregor, Miller, Blancy & nster, 1953). Among other things, there me uncertainty concerning the efficiency e washing procedures suggested. This course particularly important in cases e it is desired to assay PBI^{131} as short e as four or twenty-four hours after in- on of the tracer, because then the serum contain comparatively large quantities of ganic radioiodine.

Zieve, Vogel & Schultz (1956) state that each washing involves a loss of about 3 per cent of the PBI^{131} present. This finding has not been checked by other investigators.

In most clinical studies of PBI^{131} determination, relatively large tracer doses have been employed, *viz.* for PBI^{131} after 24 hours 50 to 100 μC, for PBI^{131} after 48 hours 25 to 30 μC (Werner, 1955).

The purpose of the present communication has been:

I: to work out a procedure permitting accurate determination of PBI^{131} after ingestion of considerably smaller tracer doses of radioiodine, *viz.* 10 to 15 μC as used in our laboratory. It has been endeavored to make the procedure not only convenient and rapid, but also reasonably accurate even in cases where the serum is relatively heavily loaded with inorganic radioiodine.

II: to estimate the accuracy of the method with particular regard to the questions of the efficiency of the washing procedure and of possible losses of PBI^{131} during the washing.

METHOD

Reagents

Trichloracetic acid (TCA) in water, 40 per cent and 20 per cent (both w/v).

Potassium iodide, 1 per cent (w/v) in water.

Procedure

About 40 ml of blood is drawn, and 12.5 ml of the serum is transferred by means of a pipette to a lusteroid centrifuge tube (16 mm by 112 mm). The pipette is conveniently filled by means of a pipetter.

If it is desired to determine the conversion ratio (Clark, Moe & Adams, 1949) 2.5 ml serum is first counted directly. Afterwards 10 ml more serum is added.

To the 12.5 ml serum, 4 ml 40 per cent TCA are added, and the mixture is stirred with a glass rod which is then removed. After a few minutes the tube with its contents is centrifuged for three minutes at 2500 r. p. m. Since lusteroid tubes tend to expand when they are subjected to hydrostatic pressure, so that they no longer fit the scintillation well counter, each centrifuge cup is provided with a hollow plexiglass cylinder inside which the lusteroid tube fits exactly. The supernatant is discarded, and one or two ml 1 per cent KI solution are squirted from a washbottle onto the precipitate, which is then stirred carefully with the rod so that a thin, uniform paste is obtained. More KI solution is added until the tube is not quite half full, and the stirring is repeated. It is important not to add too much wash fluid at once since this would cause lumps, which would make the washing procedure less efficient. The tube is almost filled up with 20 per cent TCA from another washbottle. After stirring, centrifugation is repeated. This washing procedure is performed at least three times. Samples withdrawn less than 24 hours after radioiodine ingestion are washed five times (see below).

The final precipitate is counted for ten or thirty minutes in a scintillation well counter yielding about 700 000 counts per minute for 1μC of radioiodine against a background of about 120 c. p. m.[1]

At the time of administration of a dose of radioiodine to the patient, a standard solution is prepared by dilution of an exactly identical dose with water to 100 ml in a volumetric flask. When the above precipitate is counted, 0.1 ml of standard solution is transferred by means of a Carlsberg pipette to a lusteroid tube containing 3 ml of water the volume of the standard thus obtained is approximately equal to that of the protein precipitate of the sample so that the geometry is nearly the same in both cases. The standard is counted for ten minutes.

The protein-bound radioactive iodine is expressed as the fraction of the dose ingested that is present in one liter of serum:

$$PBI^{131} = \frac{8 \text{ times the precipitate count per time u}}{\text{the standard count per time unit}}$$

per cent of dose per liter.

This formula may be derived in the following way: If the precipitate count is P and the standard count S, the count yielded by 1000 ml serum is $1000P/12.5 = 80P$. That yielded by the total dose is $100S/0.1 = 1000\ S$. The ratio of the two, expressed as per cent, is $100 \cdot 80P/1000S = 8P/S$.

In the following a few examples will be given to illustrate the order of magnitude of actual experimental values.

A well scintillation counter is not absolutely necessary. The method is practicable with any scintillation counter that can be surrounded by a lead shield such as a collimator tube used for direct measurements on the thyroid. In this case the precipitate is dissolved in about 2 *M* NaOH and transferred quantitatively to a vessel that fits the collimator bore in such a way that its bottom rests on the aluminium casing protecting the scintillation crystal. The volume is made up with water to *e.g.* 40 ml, the opening of the collimator is covered with lead, and the radioactivity is counted for thirty minutes. The standard, which consists of 0.1 ml of standard solution plus 40 ml of water, and the background are likewise counted for thirty minutes each.

Values approaching or exceeding the limit of the pathological range (0.35 to 0.4 per cent dose/l) can be determined to a relative accuracy of about 5 per cent, which is entirely satisfactory. This accuracy can be further improved by the use of 25 ml of serum for preparation of the precipitate. The smaller values found in the normal range (less than 0.1 per cent dose/l) can not be determined to a satisfactory accuracy by this procedure.

[1] The counting equipment was from Tracerlab. Inc., Boston, Mass., U. S. A.

IE EFFICIENCY OF THE WASHING PROCEDURE

the proposed method the TCA con- ration is always 10 per cent during rifugation, which eliminates the possi- of loss of protein with the supernatant. the other hand, TCA solution alone is enough to remove inorganic radioiodine. olution containing inactive potassium le is much more efficient. This is illustra- by Table I, which shows how far it is ible to remove NaI^{131} added to non- active serum.

ble I. *Removal of NaI^{131} added to non-dioactive serum by repeated washings.*

	Net counts per minute		
	Sample I washed with TCA	Sample II washed with TCA in 0.5 per cent KI	Sample III washed with TCA in 0.5 per cent KI
ml serum	84.000	84.000	9.036
ipitate (Ppt.)	16.131	16.218	1.754
washed once	4.952	2.764	459
washed twice	2.705	746	122
washed 3 times	2.030	297	68
washed 4 times	1.601	200	51
washed 5 times		194	38

amples II and III show that it is possible washing with 10 per cent TCA in 0.5 cent KI solution to remove all radio- ne except 0.2 to 0.4 per cent of the initial ntity present. After three washings there e 0.4 and 0.8 per cent left in samples II III, respectively.

he error due to the fact that it is not ible to eluate all inorganic radioiodine n the precipitate is illustrated by the fol- ng three examples, which furthermore vey an impression of the magnitude of the erimental values:

Example 1. A 24-hour precipitate counted for 10 min. Background 1298, precipitate 3777, serum 9452, standard 64640. Net counts: precipitate 2479, serum 8154, standard 63342.

$$PBI^{131} = \frac{8 \times 2479}{63342} = 0.31 \text{ per cent dose/l.}$$

Residual counts from inorganic iodine left in precipitate after three washings at most 1 per cent of 8154—2479, or about 57 counts. Thus the value of PBI^{131} found may be about 2 per cent too high. This error is slightly less than the standard deviation due to the counting.

Example 2. A 24-hour precipitate counted for 30 min. Background 3871, precipitate 5477, serum 24310, standard 258150. Net counts: precipitate 1606, serum 20439, standard 254279. $PBI^{131} = \frac{8 \times 1606}{254279} = 0.051$ per cent dose/l.

Residual counts from inorganic iodine left in precipitate after five washings at most 0.5 per cent of 20439—1606 or about 100 counts. The value of PBI^{131} may be about 6 per cent too high. Again the error is approximately equal to the standard deviation due to the counting.

Example 3. A 4-hour precipitate counted for 30 min. Background 3871, precipitate 5088, serum 72171, standard 244030. Net counts: precipitate 1217, serum 68300, standard 240159. $PBI^{131} = \frac{8 \times 1217}{240159} = 0.040$ per cent dose/l.

Residual counts from inorganic iodine after five washings at most 0.5 per cent of 68300—1217 or about 340 counts. The value of PBI^{131} may be 25 to 30 per cent too high. This is three times the standard deviation, so that it constitutes an appreciable error.

Examples 1 and 2 show that, as far as 24-hour precipitates are concerned, values approaching the pathological range can be determined with considerable accuracy, and even values in the normal range can be determined with an accuracy that is completely satisfactory. Example 3 shows that 4-hour precipitates can only be assayed very inaccurately and that the values found will always be too high.

Zieve, Vogel & Schultz (1956) found that each washing with 10 per cent TCA entails a loss of about 3 per cent of the PBI^{131} present. In an attempt to check the validity of this statement, the present author added I^{131}-labeled thyroxin to an inactive serum sample. When the proposed washing procedure was adhered to, no loss of radioactive hormone was observed.

THE CLINICAL VALUE OF PBI^{131} DETERMINATION

Determination of the protein-bound radioactive iodine can be very helpful in diagnosis of thyrotoxic conditions. Values exceeding 0.35 to 0.4 per cent dose/l for a 24-hour precipitate are usually considered diagnostic of hyperthyroidism (Werner, 1955; Friis & Christensen, 1958).

Goodwin, Mac Gregor, Miller & Wayne (1951) state that values for 48-hour precipitates exceeding 0.4 per cent suggest hyperthyroidism, and that values below 0.2 per cent may exclude this diagnosis.

The maximum normal value for a 48-hour precipitate quoted above, *viz.* 0.4 per cent dose/l, is not in accord with findings on Danish patients. In Denmark, euthyroid subjects often show higher values, but our patient material is not yet sufficiently large to permit establishment of the upper limit for the 48-hour precipitate.

The discrepancy is related to the fact that in our patients the normal thyroid uptake of I^{131} is considerably higher (up to 70 per cent of the dose) than in *e. g.* American patients.

It is important to note that the PBI^{131} may be appreciably increased even in the absence of thyrotoxicosis. This is most often true when the volume of actively functioning thyroid tissue is small. In this case the synthetic activity of the active tissue must be intense, and the hormone formed will not be stored, but will be liberated rapidly from the gland, without being diluted by preformed hormone. Such a condition involving a high PBI^{131} is found, *e. g.* in certain cases after strumectomy and in *Struma lymphomatosa* (Hashimoto).

Furthermore, a high PBI^{131} may be found in euthyroid patients treated with methylthiouracil, but only for a brief period after this treatment has been discontinued, while the gland is exhibiting a compensatory hyperactivity.

For similar reasons the PBI^{131} may be increased in iodine-deficiency goiter (Rall 1956).

All these things considered, the PBI^{131} will be one of the most valuable parameters of thyroid function in the diagnosis of thyrotoxicosis. On the other hand, determination of PBI^{131} is of no value in diagnosis of myxedema.

Clinical experience and the above reflections on evaluation of protein-bound radioactive iodine are compatible with the following expression, which may be derived from the excellent treatment of Riggs (1952):

$$PBI^{131} = k \cdot \frac{U \cdot H \cdot F_t}{V_B \cdot Q_G}$$

According to this equation the PBI^{131} will be directly proportional to the thyroid uptake of radioiodine (U), the the rate of secretion of hormonal iodine (H), and to a time factor (F_t). The magnitude of this factor depends on the rate at which inorganic iodine is lost from the organism.

The PBI^{131} will be inversely proportional to the volume of distribution of the organi

ie in the body (V_B) and to the total ntity of iodine in the thyroid gland (Q_G). e last mentioned factor is great, the I^{131}- ed hormone formed will be diluted with rge quantity of preformed hormone so the PBI^{131} will be comparatively low. reverse will be the case if Q_G is small as me cases after strumectomy or destruc- of major quantities of glandular tissue.

SUMMARY

description is given of a method for de- ination of protein-bound radioactive ne. The method permits accurate deter- ations to be done twenty-four hours after stion of a dose of radioiodine, which may educed to about 10 μC. It is stressed that nain source of error in PBI^{131} determina- is failure of the washing procedure to ove all inorganic radioiodine from the loracetic acid precipitate of the serum eins. Therefore, the efficiency of the hing procedure has been studied, and it own that efficient washing requires the of a solution containing stable iodide.

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THE RELATIONSHIP BETWEEN HYPERHEXOSAMINEMIA AND STRESS

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(Received for publication October 1, 1957)

The serum concentration of the glycoproteins and hence the hexosamines, have been reported to increase in a series of diseases of an acute and chronic nature. In general, the phenomenon of hyperglycoproteinemia seems to be unspecific. It appears, however, that the pathophysiological conditions associated with this phenomenon may be connected with an acute or chronic type of stress on the organism. Therefore it seems likely that the "stress organs" — the hypophysis and the suprarenals — may influence the metabolism of the glycoproteins. The present paper presents the results of research on serum hexosamines in rats which have been subjected to an unspecific stress after adrenalectomy or hypophysectomy, and in adrenalectomised rats after administration of ACTH. Furthermore, the results are presented of the influence on the serum hexosamine level of cortison administration combined with an unspecific stress in rats.

METHODS

Male rats weighing 250—350 g and of the same stock and age were adrenalectomised. Female rats, weighing 100—150 g and of the same stock and age were hypophysectomised. One month after the adrenalectomy or hypophysectomy, the animals, including normal controls, were subjected to an unspecific stress in the form of a 5—7 cm long incision in the skin of their backs followed by suturation. The *sella turcica* region was examined and the atrophy of the suprarenals was checked in the hypophysectomised animals after the experiment.

Female rats weighing 150—250 g and of the same stock and age were adrenalectomised. One month after the adrenalectomy the animals, including normal controls, were exposed to an unspecific stress, and ACTH, 1 mg dissolved in 0.2 ml of distilled water, was administered intramuscularly twice daily to 3 of the normal and 3 of the adrenalectomised animals over a period of 4 days. In order to evaluate the possible stress caused by the injection itself, the animals were subjected to another experiment one month later. They were divided in two groups, both consisting of normal as well as adrenalectomised animals. ACTH in the same doses as mentioned above was administered to the rats in one of the groups, the other animals were simultaneously given corresponding doses of distilled water intramuscularly.

Male rats, weighing 195—250 g and of the same stock and age were treated daily with 10 mg cortisone acetate intramuscularly over a period of 9 days. On the 6th day of the cortisone administration all the animals, including non-treated control animals, were subjected to an unspecific stress. Blood samples, for the determination of total protein and hexosamines, were obtained by heart puncture in ethyl ether anesthesia before and after the experiments. The serum content of hexosamines was determined according to a modification of the Elson-Morgan procedure as previously described by the present author (Rosenlund, 1956) and the total protein by the biuret method (Kingsley, 1939). The hexosamine content is expressed as a percentage of total protein. (Tables I, II, III, IV.)

le I. *Serum content of protein-bound amines in adrenalectomised and hypophysectomised rats following an unspecific stress*

nals	Hexosamines in per cent of total protein		Difference in per cent	Mean difference
	Before stress	After stress		
alectom.				
	2.16	2.70	+25.0	
	2.23	2.56	+14.8	+15.5
	2.17	2.33	+ 6.9	
hysect.				
	1.99	2.50	+25.3	
	1.78	2.34	+31.4	+18.1
	2.04	2.24	+ 9.8	
	2.12	2.24	+ 5.7	
ls				
	2.29	2.34	+ 2.6	
	2.12	2.68	+26.4	
	2.15	2.60	+21.0	+14.9
	1.91	2.18	+14.1	
	1.92	2.12	+10.4	

le II. *The effect of ACTH on the n protein-bound hexosamines in normal adrenalectomised rats subjected to an unspecific stress*

ormal animals a = adrenalectomised animals

mals	Hexosamines in per cent of total protein		Difference in per cent	Mean difference
	Before stress	After stress		
s + H				
n	1.86	2.50	+34.4	
n	2.01	2.59	+14.0	
n	1.94	2.44	+25.8	+23.9
a	2.04	2.44	+19.6	
a	2.13	2.56	+20.7	
a	2.00	2.58	+29.0	
s only				
n	1.93	2.03	+ 5.2	
a	2.00	2.25	+12.5	+ 6.4
a	2.14	2.17	+ 1.4	

Table III. *The effect of ACTH compared to the effect of distilled water administred intramuscularly on the serum protein-bound hexosamines in normal and adrenalectomised rats*

n = normal animals a = adrenalectomized animals

Animals	Hexosamines in per cent of total protein		Difference in per cent	Mean difference
	Before injections	After injections		
ACTH				
n	1.96	2.22	+13.2	
a	2.09	2.42	+15.8	+16.5
a	2.11	2.54	+20.4	
Distilled water				
n	2.01	1.98	— 1.5	
n	2.05	1.89	— 7.5	— 3.2
a	2.16	2.15	— 0.5	
a	2.08	2.01	— 3.4	

Table IV. *Serum content of protein-bound hexosamines following treatment with cortisone in rats subjected to an unspecific stress*

Animals	Hexosamines in per cent of total protein		Difference in per cent	Mean difference
	Before stress	After stress		
Cortisone-treated				
1	1.91	2.04	+ 6.8	
2	1.87	2.00	+ 7.0	+ 6.9
3	2.14	2.29	+ 7.0	
Controls				
1	2.03	2.44	+20.2	
2	1.89	2.17	+14.8	+17.5
3	1.83	2.15	+17.5	

RESULTS

It appears from Table I that the adrenalectomised as well as the hypophysectomised animals, following an unspecific stress, show an increase in the serum hexosamine concentration which is of the same order as that observed in the control animals. No remnants of the hypophyses were found on gross examination of the *sella turcica* region of the hypophysectomised animals. The mean weight of the suprarenals from the hypophysectomised animals was 10 mg, from the control animals 52 mg.

The animals which were subjected to stress in combination with ACTH administration exhibited a much more pronounced rise of the serum hexosamine content than the animals subjected to stress only, whether they were adrenalectomised beforehand or not (Table II). It appears from Table III that ACTH alone produced a pronounced increase of the serum hexosamine concentration. The effect of ACTH cannot be ascribed to the minor stress caused by the intramuscular injections, as the animals which were given distilled water intramuscularly did not show any increase of the serum hexosamine content. From Table IV it appears that the hyperhexosaminemia was less pronounced in those rats which were treated with cortisone in addition to unspecific stress than in those animals which were subjected to stress only.

Even if the material for these experiments is limited, the observations indicate that the hyperhexosaminemia observed during an unspecific stress in the form of a minor surgical trauma cannot be due to the hyperfunction of the suprarenals observed under such circumstances.

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Scand. J. Clin. & Lab. Investigation 219—220, 10, 1958.

THE ROLE OF THE LIVER IN STRESS HYPERHEXOSAMINEMIA

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(Received for publication October 1, 1957)

he mechanism of the hyperglycopro-
mia and hence the hyperhexosaminemia
rved during stress is not known. Gersh
Catchpole (1949) have suggested that
genases may depolymerize the poly-
harides of the tissue glycoproteins, thus
easing the serum content of these sub-
ces. Kushner, Honig, Dubin, Dyniewicz,
sky, de La Huerga & Popper (1956)
ests that "stress may act either by the
of an adrenal influence on serum pro-
formation or may in itself cause tissue
kdown, with the resultant products
ng as components of the serum muco-
in". Werner (1949), on the other hand,
rted that the regeneration of serum
osamine takes place much faster than
regeneration of the serum proteins after
icial bleeding of rabbits. The regenera-
of glucosamine, however, did not take
if the animals were given liver poisons
as phosphorus or benzene perorally.
ner suggests that glucosamine is syn-
sed in the liver.

order to investigate the role of the liver
mechanism of the hyperhexosaminemia
ng stress, rats subjected to poisoning by
phorus were exposed to an unspecific
s or injected with ACTH.

METHODS

Male rats weighing 200—300 g were used. To 3 of the animals was administered 0.1 ml of a solution of 0.25 per cent of phosphorus in liquid paraffin by a stomach tube on 3 successive days. The animals were then subjected to an unspecific stress (incision of the skin of their backs followed by suturation) and the administration of phosphorus was continued for 3 more days.

ACTH, 2 mg twice daily, was administered to 2 animals for a period of 4 days. From then on 0.1 ml of the phosphorus solution was administered daily perorally for a period of 4 days, and the injections of ACTH were continued for the same period. Blood samples for the determination of hexosamines were obtained by heart puncture in ethyl ether anesthesia before and after the experiments. Hexosamines were determined according to a modification of the Elson-Morgan procedure previously described by the present author (Rosenlund, 1956).

RESULTS

It appears from Table I that an increase of the serum hexosamine content takes place in the control animals following unspecific stress or ACTH-administration, whereas the serum content of hexosamines *decreases* in the animals which were subjected to poisoning by phosphorous.

Even if split protein products from tissue breakdown may be assumed to act as a

Table I. *Serum content of protein-bound hexosamines following an unspecific stress and administration of ACTH, respectively, in rats subjected to poisoning by phosphorus.*

Animals	Hexosamines in mg/100 ml		Difference in per cent	Mean difference
	Before experiment	After experiment		
Phosphorus + stress				
1	132	102*	— 22.7	
2	140	109	— 22.1	
3	136	102	— 25.0	
Phosphorus + ACTH				— 22.9
1	139	128	— 7.9	
2	149	94	— 37.0	
Controls				
1 (stress)	139	148	+ 6.5	
2 (stress)	122	141	+ 15.5	
3 (stress)	129	139	+ 7.7	+ 9.6
4 (ACTH)	149	162	+ 8.7	

* After one more day 71 mg/100 ml.

source of the increased protein-bound polysaccharides in stress, as suggested by Gersh & Kushner, such a mechanism cannot be regarded important, as rats, subjected to a surgical trauma during poisoning with phosphorus showed a decrease of the serum hexosamine content. As a microscopic examination of the liver of one of the poisoned animals showed fatty infiltration, degeneration of the liver cells and karyolysis and karyorrhexis of some of the nuclei, the lowered hexosamine values in these animals may be regarded as a result of the liver damage caused by the phosphorus. The experiments thus indicate that the liver plays an important role in the mechanism of hyperhexosaminemia during stress.

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Technique and Practical Problems

inav. J. Clin. & Lab. Investigation 221—223, 10, 1958.

DETERMINATION OF RESPIRATORY GASES BY PARAMAGNETIC OXYGEN ANALYSER AND CARBON DIOXIDE CATHAROMETER

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(Received for publication December 11, 1957)

cardio-respiratory laboratories there been an increasing need of rapid and t methods for determination of respira- gases. For several years instruments for mination of oxygen and carbon dioxide, arily designed for industrial use, have available. As the adaption of these in- nents for physiological work presents in problems, we have thought it to be terest to publish our experience with a magnetic oxygen analyser and a carbon ide catharometer.[1]

METHODS

Instruments

ramagnetic oxygen analyser. Oxygen is attrac- by magnetic fields. How this paramagnetic ptibility, almost unique among common gases, ilized in the oxygen analyser, is described here. (Catton, 1957.)

ne instrument contains a pressure sensitive which compensates for variations in atmos- c pressure by changing the temperature of the tor, and thereby the paramagnetic suscepti- . This permits calibration of the indicator in ne per cent of oxygen.

ne detector is slightly sensitive to the composi- of the gas with which oxygen is mixed, prob- dependant on the thermal conductivity of the paramagnetic gases. Five volume per cent of n dioxide causes a response corresponding to olume per cent of oxygen.

The sensitivity to water-vapor is negligible. The response is practically independant of the flow rate through the analyser, apart from the influence of flow on the time-lag. A steady reading could be obtained in two minutes at a flow of 150 ml/min.

Carbon dioxide catharometer. The instrument measures the thermal conductivity of gases entering a detector unit in the catharometer by diffusion (Comroe, 1950). This principle is particularly well suited for determination of gases with high thermal conductivity and diffusibility, such as hydrogen and helium. For measurement of gases like carbon dioxide, which has a thermal conductivity of about one-hundreth of that of helium, the instrument has to be very sensitive, and the time needed for equilibration is prolonged.

Procedure

Each determination was started by checking the instruments with carbon dioxide-free, water-vapor-saturated atmospheric air. The gas to be analysed was pumped through the catharometer at a rate of 400 ml/min., and thence was led to the oxygen analyser through a soda lime absorber which removed carbon dioxide and water vapor. The equilibration time of the catharometer was 15 minutes, but for practical reasons the carbon dioxide indicator was read after exactly two minutes. The reading thereby obtained was 95 per cent of the reading after equilibration.

In our set-up the reading of the oxygen indicator was stable after 3 minutes.

CALIBRATION

The paramagnetic oxygen analyser was calibrated against mixtures of oxygen and nitrogen, measured on the *Haldane* gas analysis apparatus.

Cambridge Instrument Co. England. No. 5 and 564341. The instruments have been ied by grants from The Danish State Research lation.

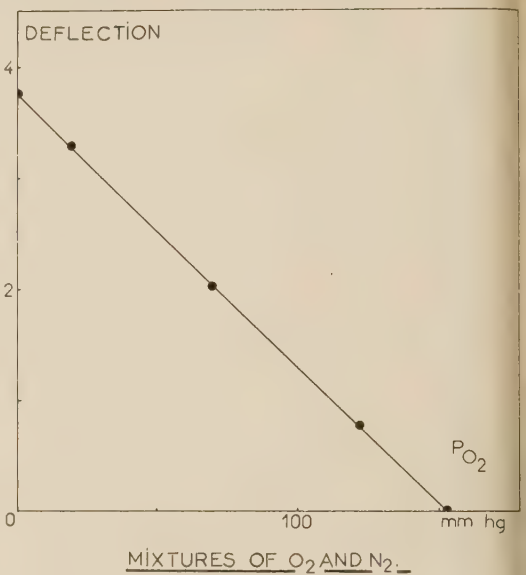
The carbon dioxide catharometer was calibrated against mixtures of carbon dioxide and atmospheric air by the factory. For determination of carbon dioxide in expiratory air this calibration cannot be used, as the deflection of the indicator depends not only on the carbon dioxide content but on the amount of oxygen and nitrogen in the particular mixture as well. The error committed by neglecting the difference in oxygen and nitrogen concentrations of expiratory and atmospheric air, *i. e.* by using the original calibration, amounts to about 1 volume per cent of carbon dioxide. The correction to be applied must allow for variations in the concentration of all three gases.

This difficulty may be coped with in different ways. Holmgren (1954) made an empirical calibration, using mixtures of the gases in the concentration interval in which most values were expected to be found. We have used a more theoretical approach which has the advantage that the instrument can be used for determination of other gases and in greater concentration intervals.

The deflection of the carbon dioxide indicator depends on the thermal conductivity of the expiratory gas compared with that of carbon dioxide-free, water-vapor-saturated atmospheric air which is used for setting of the zero of the catharometer. The deflection therefore may be regarded as the sum of thermal conductivities of the component gases, *i. e.*

$$G = x \times pCO_2 + y \times pO_2 + z \times pN_2 \quad (1)$$

where G is the deflection and x, y and z are constants representing the relative thermal conductivities (in scale units) caused by 1 mm of mercury of each gas. These constants were determined by different mixtures of two of the three gases at a time. Figure 1 shows the rectilinear relationship between deflection of the indicator and variation of the oxygen and nitrogen tension of mixtures of these two gases, determined on the Haldane apparatus. From this curve the values of y and z were calculated by extrapolation.



MIXTURES OF O_2 AND N_2.

A similar curve for calculation of x was constructed by using mixtures of carbon dioxide and nitrogen.

CALCULATIONS

The oxygen tension in the gas pumped through the carbon dioxide catharometer can be expressed from the reading on the paramagnetic analyser F (volume per cent) after correction for absorbed carbon dioxide and water vapor as

$$p_{O_2} = \frac{F}{100} (p_B - p_{H_2O} - p_{CO_2}) \quad (2)$$

The nitrogen tension in the same mixture is

$$p_{N_2} = p_B - p_{H_2O} - p_{O_2} - p_{CO_2} \quad (3)$$

The values for p_{O_2} and p_{N_2} can be inserted in (1). The solution

$$p_{CO_2} = \frac{G - (p_B - p_{H_2O}) \cdot \left(\frac{F}{100} (y - z) + z\right)}{(x - z) - \frac{F}{100} (y - z)}$$

makes calculation of p_{CO_2} easy for routine use when corresponding values of F and F/100 (y—z) are tabulated.

le I. *Standard deviations in analysis of ent mixtures of oxygen, nitrogen, and carbon dioxide.*

ygen 00 ml	Carbon dioxide ml/100 ml	Standard deviation Oxygen	Carbon dioxide
.94	0.00	0.05	0.04
.91	3.00	0.03	0.01
.69	4.81	0.03	0.04
.64	6.46	0.03	0.03

ANALYTICAL ERROR

e divisions of the scales of the indicators t readings with an accuracy of 0.05 ie per cent of oxygen and 0.02 volume ent of carbon dioxide in the range used hysiologic work.

e analytical error of the instruments was ed by ten determinations carried out at of a number of mixtures of oxygen, n dioxide, and nitrogen. The results are in Table I.

From these figures it may be estimated that the analytical error of a typical determination of oxygen uptake is about 1—2 per cent.

CONCLUSIONS AND SUMMARY

The use of a paramagnetic oxygen analyser and a carbon dioxide catharometer in respiratory physiology is described, with special reference to their calibration. The advantage of using these instruments is chiefly that of time saving, which must be balanced against the draw-backs of greater analytical errors, compared to conventional chemical methods, of greater amounts of gas necessary, and of the cost of the equipment.

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Scandinav. J. Clin. & Lab. Investigation 224, 10, 1958.

A KYMOGRAPH DRIVEN BY A SYNCHRONOUS MOTOR WITH A CONTINUOUSLY VARIABLE VELOCITY

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(Received for publication December 17, 1957)

The object of this paper is to draw attention to the synchronous motor type of velocity control. If such a motor is driven by a sinusformed AC with a variable frequency the velocity of the motor will be exactly proportional to the frequency. Such a variable AC can be achieved from a tune generator, which should cower the range of about 15—300 cycl/sec and be vary stable in frequency (at least 0.5 per cent after burning in). The period number of the generator is varied in the usual manner by a knob with a large dial and scale as seen in Fig. 1. The output of the tune generator is fed into a power amplifier inside the generator which allows for an output of 50 w. The scale should have period number and the corresponding surface velocity of the kymograph plotted simultaneously. Most synchronous motors can not to advantage use the full range of 15—300 cycl/sec, but something like 20—200. They must in any case be started at about 50 cycles. A kymograph driven by a synchronous motor can be fed from a generator with variable frequency and then aquires the quality of continuously variable velocity over the range 1 : 10.

COMMENTS

This system offers certain advantages, namely the following:

1. The exact velocity can easily be remotely controlled and the handling can also without difficulty be made automatic so as to follow a definite schedule. This is of value in experiments of long duration that may need both a certain continuous graphic control as also, at intervals, a more or less extended curve.

2. In experiments checked from outside a closed room (*e. g.* an under-pressure-chamber) one can observe the course of a recordin and adapt the velocity to the immediate nee

3. Another example is the investigatio of the frequency response of a recording in strument. A writing manometer is to b tested for its ability to render different fr quencies correctly. This can be done in suitable manner if the instrument is supplie with a sinusformed pressure variation fro a piston arrangement with variable velocity The manometer is writing on a synchronou kymograph (Fig. 1). If both kymograph an piston motor are fed from the same variabl AC generator, both will vary at the sam range and it is therefore possible to recor in a constantly symmetrical recording syste where the proportion between abscissa an ordinate is the same at different frequencie Not only the amplitudes but also the shap of the curves are thus directly comparabl

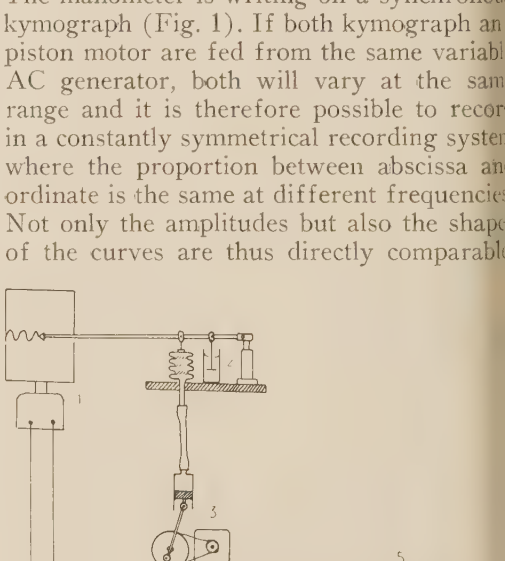

Fig. 1. Arrangement for investigating the frequen response of an oil-dampened recording manomete
1. Kymograph driven by a synchronous motor.
2. Recording manometer with oil-dampening.
3. Piston driven by a synchronous motor.
4. Power amplifier max. 300 volt, 50 watts.
5. Tune-generator 15—300 cycl/sec.

linav. J. Clin. & Lab. Investigation 225—226, 10, 1958.

BAG SPIROMETER

By P. F. SCHOLANDER and H. JENSEN

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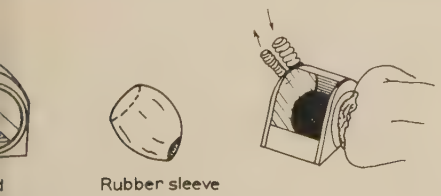
(Received for publication December 12, 1957)

ıring a recent field investigation on cold natization in Australian aboriginals, it desirable to measure the oxygen con- tion of subjects sleeping throughout the naked on the ground. This called for pen circuit system, where the person rest his head on a pillow inside a ven- l hood (Fig. 1). An air-tight connection e hood was obtained by means of an thinwalled rubber sleeve, made by ıg both ends off a football-sized mete- gy balloon. One end fitted over the e of the hood, the other fitted with its rsized hole airtight around the neck. arrangement permitted the subject to his head unrestrained, and he could on either side.

tdoor air was drawn through the hood eans of a battery driven blower. The t was regulated by means of a valve ı low resistance flow meter to about 0 l/min, which gave some 0.5—1 per CO_2 in the hood. The air then passed bag-spirometer (Fig. 2). This consists y of two circular plywood end plates, shed and covered with aluminum foil e inside, and sealed to a thin cylindrical



Respiratory hood and detached rubber sleeve r studying gas exchange during sleep.

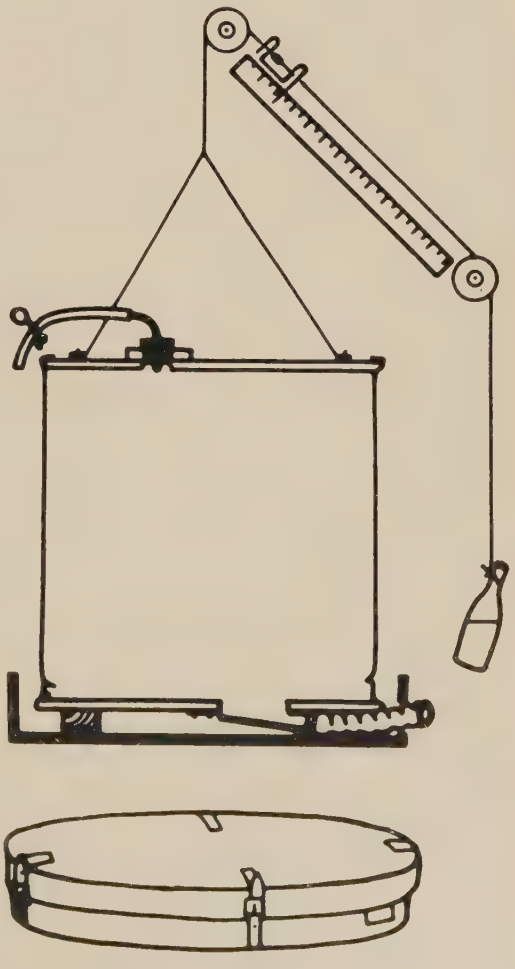


Fig. 2. Bag spirometer. Below, spirometer in portable carrying case.

plastic bag which is made by welding together two sides of a 0.16 mm thick polyvinyl chloride sheet, accurately cut to a rectangle. The bag, stretched over the rims of the end plates, is sealed to these by adhesive tape. The bottom plate has an inlet pipe, and the top plate a plastic square with a hole for a rubber stopper, through which the gas-sampling tube passes. The lid hangs by three wires and a string which passes over two pulleys (airplane sheaves) to the counterweight. For this a liter bottle is suitable containing sufficient water to maintain an overpressure in the closed bag so it will stand up with taut walls.

When the spirometer is being inflated or deflated the bag usually wrinkles only at the bottom. It rises and falls just like an ordinary spirometer, and meters the gas volumes very accurately. The gas volumes are conveniently read by letting a wire pointer ride loose on the counter weight string, prevented from sliding down by an adjustable stop. Before reading the spirometer it is wise to stroke the sides downward and collect all folds at the base. This also serves to mix the gas. With this precaution our 400 liter spirometer bag metered within half of 1 per cent, *i. e.*, within 2 liters. It is often desirable to have a thermometer, or a thermocouple, passing through the lid stopper, or through the lid itself. A calibration curve is given in Fig. 3, where a 400 liter bag spirometer was fed from an ordinary Tissot--type spirometer.

Gas tightness of the system is checked by letting it stand closed, and reading the scale. Tests on diffusion tightness for oxygen and CO_2 revealed, under the worst conditions, with the spirometer almost empty, no detectable diffuson loss of CO_2 or oxygen during an hour's time, when the gas mixture contained about 1 per cent CO_2 and 20 per cent oxygen. Analyses were made with the $^1/_2$ cc analyzer and checked within 0.02 per cent. It should be stressed that the diffusion tightness depends entirely upon the material used in the bag. Polyethylene, for instance, cannot be used.

The calibration of the bag, which is near

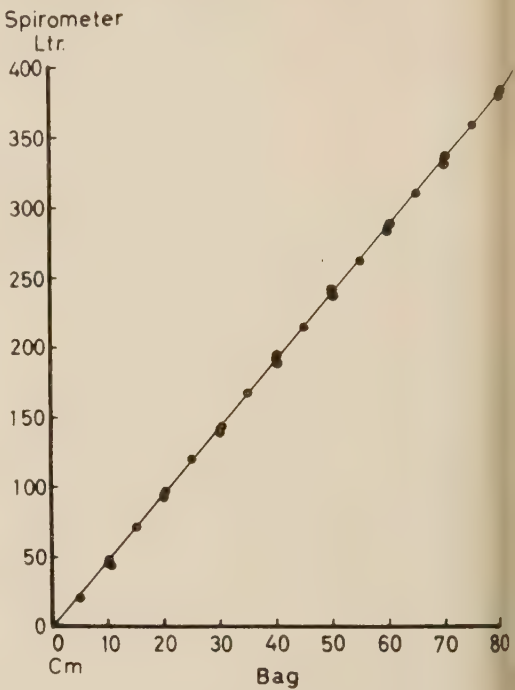


Fig. 3. Calibration curve of 400 l bag spirometer

a cylinder, can be checked in the field simply by measuring the circumference of it with a length of 2-inch wide gauze. If a piece of wire is attached to each end of this by adhesive tape it can easily be stretched loosely around the bag to serve as a tape measure. The volume calibration, calculated from the circumference of the bag and directly measured by an accurate conventional spirometer, checked within a half of one per cent. It did not change as a result of the long transport from Norway to Australia, and remained exactly the same throughout our use of the spirometer in the desert.

The merits of the system are its light weight and small bulk. A 500-liter spirometer weighs about 5 kg and can easily be carried as airplane baggage.

Metering bag-spirometers of some 80 liters' capacity were used by the senior author some 10 years ago for preparing large quantities of known carbon monoxide-air mixtures.

Letters to the Editors

linav. J. Clin. & Lab. Investigation 227—228, 10, 1958.

THE EFFECT OF THE PHOSPHOLIPIDS OF DAIRY PRODUCE ON THE RECALCIFICATION TIMES OF HUMAN PLASMA

any workers have shown the effect of meals, and of dairy fats, on the clotting of the blood (O'Brien, 1956, MacLagan llimoria, 1956).

e were interested in the effects of the from different forms of dairy produce hole plasma, and as lipids can influence ecalcification times of plasma, this test ised throughout as the indicator of lipid ity. It appeared that in this estimation coagulation system was as natural as ble.

METHODS

agulation tests. Recalcification times were ated as decribed elsewhere (Kingsbury & an, 1957).

sma. In all tests fresh human plasma was Fasting plasma was collected before breakfast an overnight fast and fat-free supper, and ic plasma 3 hours after a meal of 40 g of dairy produce.

ffer. 0.04 M veronal buffer of pH 7.4 was hroughout.

aporations. All evaporations were carried out reduced pressure using only sufficient heat t the ice which formed on the flask. Excessive ng was prevented by bubbling CO_2 through lution and the flask was shielded from direct

EFFECT OF DAIRY PRODUCE ON HE RECALCIFICATION TIMES OF HUMAN PLASMA

rial dilutions in buffer of 1:2, 1:10 1:20 were made using fresh double n, whole milk, skimmed milk and butter. l of each of these dilutions was added ml of fasting plasma and to 1 ml of nic plasma and the recalcification times

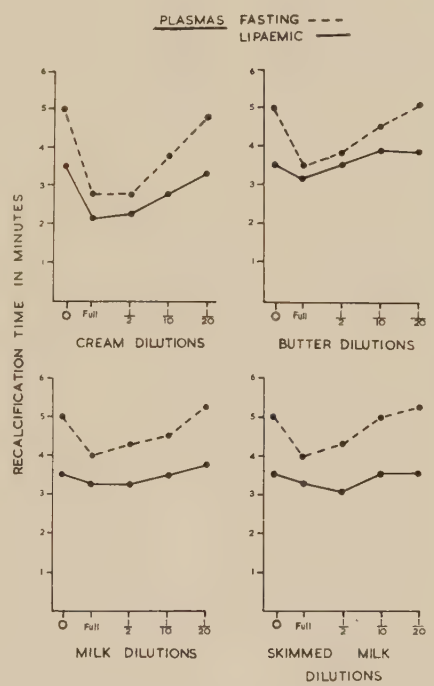


Fig. 1. The effect of dairy produce on the recalcifiation times of human plasma.

observed. It was found (Fig. 1) that although all the produce tested accelerated coagulation, the effect was not proportional to the neutral fat content.

It appeared likely that the activity might be due to the phospholipids; to investigate their possible importance butter was fractionated according to the flow diagram (Fig. 2).

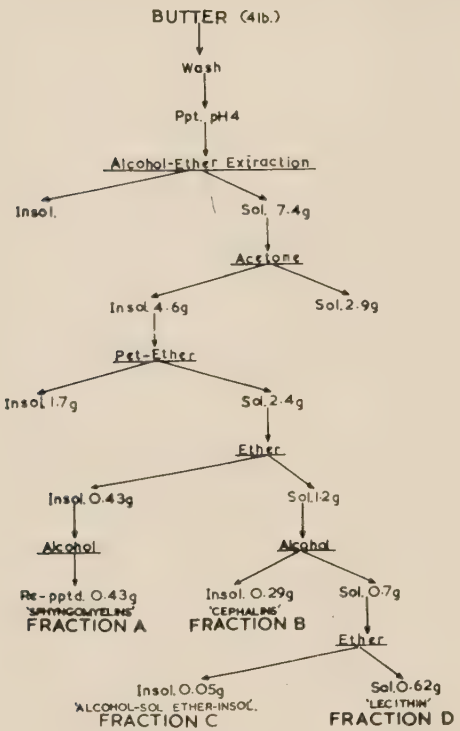


Fig. 2. Flow diagram of the fractionation of butter-phospholipids.

The various fractions were taken up in veronal buffer to give final concentrations of 50 mg/100 ml. 0.1 ml of each of these suspensions was added to 1 ml of the plasma (*i. e.* an addition of 0.05 mg of material) and the recalcification times observed. The results are given in Table I and are the averages of consistent series (Kingsbury & Morgan, 1957) obtained using materials prepared from four separate butter fractions. The "alcohol-soluble ether-insoluble" fraction was always very active, followed by the "cephalins"; the "sphyngomyelins" and "lecithins" had very little effect.

Surgical Unit,
St. Mary's Hospital,
London,
England.
December 12, 1957.

Table I. *The effect of butter-phosph lipids on plasma recalcification times.*

Fractions			
A	B	C	D
3 min 42 sec	3 min 10 sec	4 min 40 sec	4 min 10 se

Controls
4 min 38 sec

DISCUSSION

These experiments confirm that whe dairy produce is fractionated only the pho pholipids accelerate the recalcification time of which the most active appears to be fra tion C, obained from the "lecithins" by pr ipitation with ether. The composition of th fraction is unknown but we are preparin larger quantities for further investigatio

The minimum quantity of diary produc which accelerated the recalcification time corresponded to an addition of about 5 m of phospholipid per 100 ml of plasma. Th is particularly interesting for it correspond to the minimal amount of the phospholipi from chylomicrons required to affect recalc fication times (Kingsbury & Morgan, 1957)

SUMMARY

It has been shown that dairy produce a celerates the coagulation of human plasm *in vitro*, measured by recalcification test and that the action is due to the phospholipi content of which the "cephalin" group an an "alcohol-soluble ether-insoluble" pho phatide were most active.

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K. J. Kingsbury,
D. M. Morgan.

dinav. J. Clin. & Lab. Investigation 229—230, 10, 1958.

UDIES ON A δ-AMINOLEVULIC ACID-LIKE SUBSTANCE IN URINE FROM LEAD WORKERS

n an earlier investigation (1957) it was wn that the excretion of a δ-aminolevulic -like substance (X) in the urine was n markedly increased among lead kers.

he method of analysis devised by Mau- ll & Granick (1956) for determination δ-aminolevulic acid (ALA) is based on fact that, when heated with acetylacetone, A condenses to a pyrrole, which with methylaminobenzaldehyde (DMAB) in solution forms a colored complex that be determined spectrophotometrically. wever, since also other aminoketones and osamine, on exposure to acetylacetone, d condensation products reacting with AB to form colored complexes, it was sidered desirable to form a clearer con- of the composition of X, *i. e.* whether it ALA or a related substance that had found in abnormal amounts in urine n many lead workers.

MATERIAL

he material consisted of urine from a lead er with a daily excretion of about 0.03 mg/ml alculated as ALA. X was concentrated in the wing way: 200 ml of this urine, pH 6.2, was ed through two resin columns, Dowex 2-X8 Dowex 50-X8, prepared according to Mauzerall ranick. The volume of each column was about m[3]. After the urine had been passed through Dowex 2-X8 column, this was washed with ml of distilled water. The urine and the water mixed and passed through the Dowex 50-X8 nn. The urea retained in this column was ved by rinsing with distilled water, after which as eluated with 100 ml of 0.5 M sodium acetate. eluate was collected in 3 ml fractions. The ntration of X in each fraction was determined rding to Mauzerall & Granick, after which the ion with the highest concentration was used he investigations, the results of which are reported below. This solution of X had a concentration of about 0.5 mg/ml, when calculated as ALA.

RESULTS

1. Two dimensional paper chromatography (phase 1: butanol-glacial acetic acid-water, phase 2: saturated aqueous solution of phenol) gave, after development with ninhydrine, a spot of the same color and Rf value as pure ALA[1]-HCl-solution under corresponding conditions.

2. Fig. 1 shows the spectra of the condensation products between a/ substance X and DMAB and b/ ALA-HCl and DMAB. X and ALA-HCl had been heated with acetylacetone before the condensation with DMAB. These light absorption spectra are identical.

3. δ-aminolevulic acid dehydrase (ALAD) was prepared according to Gibson, Neuberger & Scott (1955). A concentrated solution of substance X and glutathione was incubated with this enzyme. A colored product was obtained with DMAB after this incubation, but not before.

The spectrum of this product, after reaction with DMAB, was identical with that of porphobilinogen (PBG) (Fig. 2). PBG had been isolated by the method of Cookson & Rimington (1954) from urine from patients with acute porphyria.

4. Grieg, Askevold & Sveinsson (1950) have shown that PBG can be condensed to porphyrin *in vitro*. When substance X was treated first with ALAD and then in the way described by these authors, porphyrine was obtained.

These four experiments strongly suggest that the ALA-like substance found in in-

[1] We wish to thank Parke, Davis & Co., Detroit, Mich. for courteously supplying ALA-HCl.

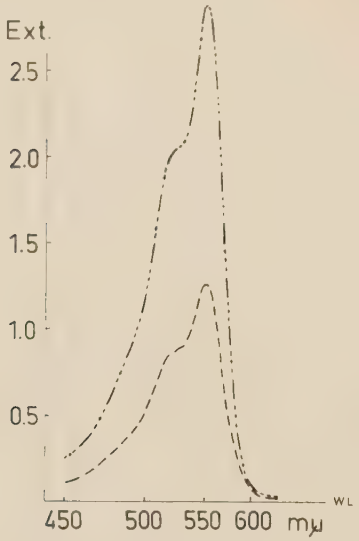

Fig. 1. Spectral analysis in Beckman DK
Condensation products between:
– – – – – ALA-like substance, heated with acetylacetone and subsequent reaction with DMAB.
—·· —·· ALA-HCl, Parke, Davis & Co., heated with acetylacetone and subsequent reaction with DMAB.

creased quantity in the urine from lead workers consisted at least mainly of δ-aminolevulic acid.

The experiment will be presented in greater detail in a future paper.

Department of Clinical Chemistry
Malmö General Hospital,
Malmö, Sweden.

February 2, 1958.

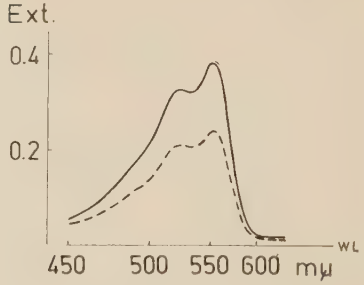

Fig. 2. Spectral analysis in Beckman DK
Condensation products between:
– – – – – Compound formed on incubation o ALA-like substance, glutathione an ALAD and subsequent reaction wit DMAB.
– – – – – Phorphobilinogen and DMAB.

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THE URINARY EXCRETION OF PARENTERALLY ADMINISTERED RADIOVITAMIN B_{12} IN RENAL FAILURE

The introduction of Schilling's urinary oactivity test (URT) (Schilling, 1953) considerably facilitated the clinical diagis of pernicious anemia. Owing to its plicity and rapidity, this test has many antages as compared with the other radiomin B_{12} absorption tests used for routine poses. However, since it is the urinary retion that is measured, the URT is congent on a normal kidney function. Recent estigations (Rath, McCurdy & Duffy, 7) as well as our own experience indicate in renal failure low URT values are nd, the value often being in the pernicious mia range, *i. e.* lower than 5 per cent. The excretion test has likewise been found give unreliable results in renal failure uhlbäck & Wahlberg, 1958). In order to cidate whether the low radiovitamin B_{12} retion values observed in renal failure are to impaired renal excretion or to deased intestinal absorption of B_{12}, the al excretion of parenterally administered Co^{60} has been followed in normal subjects in patients with renal failure.

MATERIAL AND METHODS

Kidney function and $B_{12}Co^{60}$ excretion were stuin 10 control subjects and 10 patients with l failure in a relatively steady state. Endogenous tinine clearance, performed in the conventional ner, was used as the principal kidney function The clearance was followed for two 2-hr. ods, and the values were corrected to correspond body surface of 1.73 square meters. The Folin ate method, as modified by Brod & Sirota 8), was used for creatinine determination.

A sterile solution containing 1002 μg $B_{12}Co^{60}$ was injected intramuscularly into the test subjects, and the urine was subsequently collected for two 24 hr. periods. The low specific activity radiovitamin B_{12} was prepared by mixing 1000 μg of nonradioactive B_{12} with 2 μg of $B_{12}Co^{60}$ with a specific activity of 150 μc/mg.

RESULTS AND DISCUSSION

As seen in Tables I and II and Fig. 1, the urinary excretion of $B_{12}Co^{60}$ was considerably decreased in renal failure, especially during the first 24 hours. This decrease was independent of the urinary volume. Rath, McCurdy & Duffy (1957) found that administration of intrinsic factor failed to raise the low URT values found in renal failure. The present study also supports the assump-

Table I. *The excretion of parenterally administered $B_{12}Co^{60}$ in the control subjects.*

Case no.	Endogenous creatinine clearance ml/min.	$B_{12}Co^{60}$ excretion as percentage of parenteral dose		
		0—24 h	24—48 h	0—48 h
1	137	70.6	1.1	71.7
2	129	73.8	0.9	74.7
3	120	86.4	0.4	86.8
4	111	80.4	1.2	81.6
5	102	71.1	1.7	72.8
6	95	64.5	1.3	65.8
7	95	51.2	0.1	51.3
8	82	67.5	0.5	68.0
9	81	63.5	1.8	65.3
10	78	82.7	0.8	83.5

Table II. *The excretion of parenterally administered $B_{12}Co^{60}$ in renal failure.*

Case no.	Diagnosis	Endogenous creatinine clearance ml/min.	$B_{12}Co^{60}$ excretion as percentage of parenteral dose			Mean daily urine output ml
			0—24 h	24—48 h	0—48 h	
11	Chronic pyelonephritis	36	51.4	4.0	55.4	2300
12	Chronic nephritis	29	26.8	4.3	31.3	1300
13	Chronic pyelonephritis	27	23.8	1.0	24.8	1700
14	Nephrosclerosis	13	24.0	14.2	38.2	700
15	Chronic pyelonephritis	11	26.2	12.5	38.7	800
16	Chronic nephritis	11	26.2	8.0	34.2	2600
17	Chronic nephritis	11	27.6	5.0	32.6	1200
18	Chronic pyelonephritis	10	27.6	13.5	41.1	1100
19	Chronic nephritis	7	17.2	12.4	29.6	2100
20	Nephrosclerosis	3	11.0	9.3	20.3	700

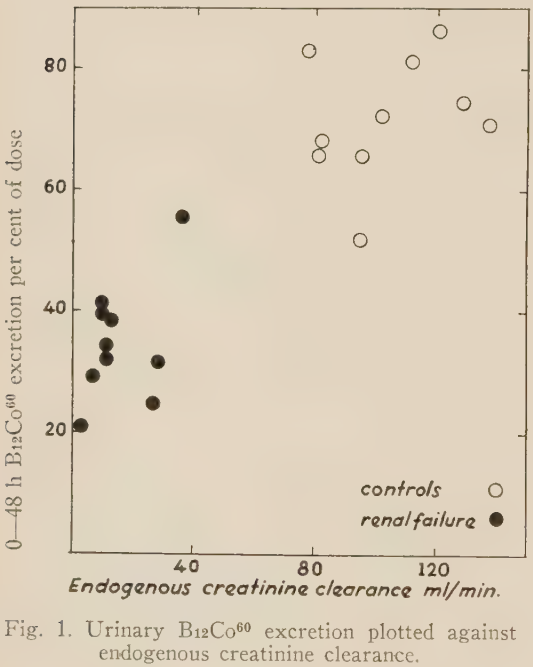

Fig. 1. Urinary $B_{12}Co^{60}$ excretion plotted against endogenous creatinine clearance.

tion that the low URT values found in uremia are more probably dependent on decreased glomerular filtration than due to impaired intestinal absorption of vitamin B_{12}.

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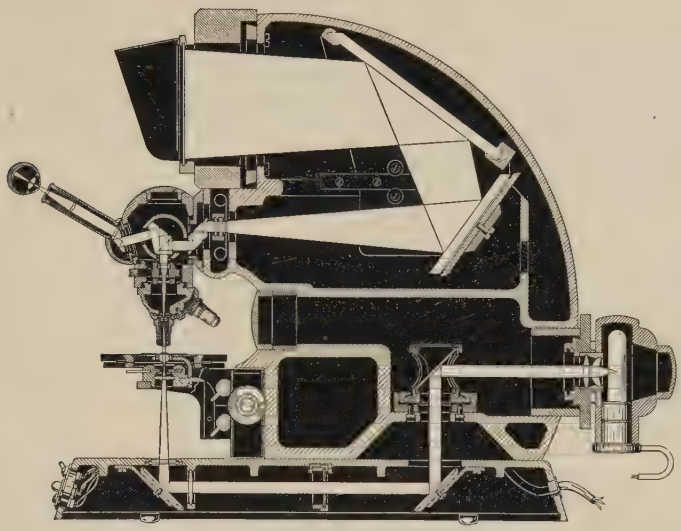

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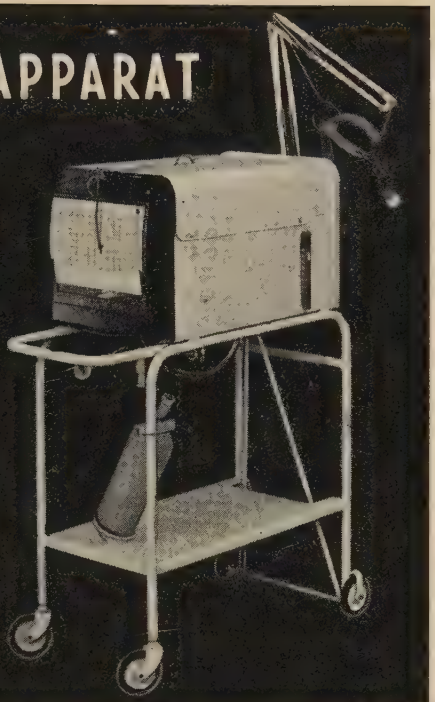

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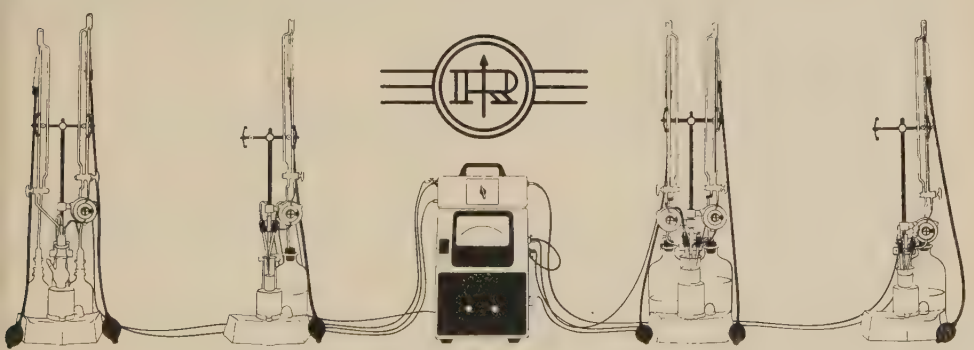

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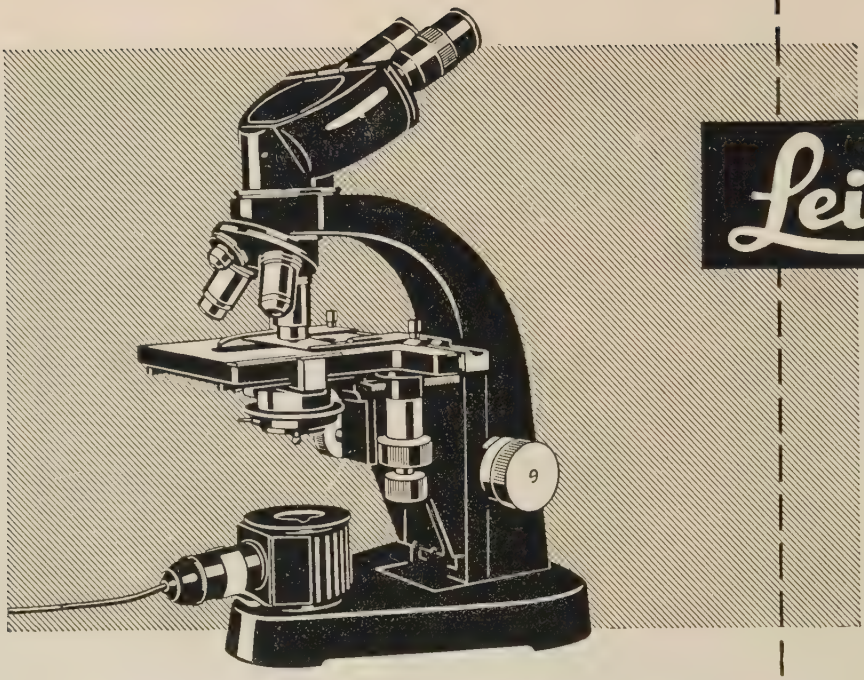

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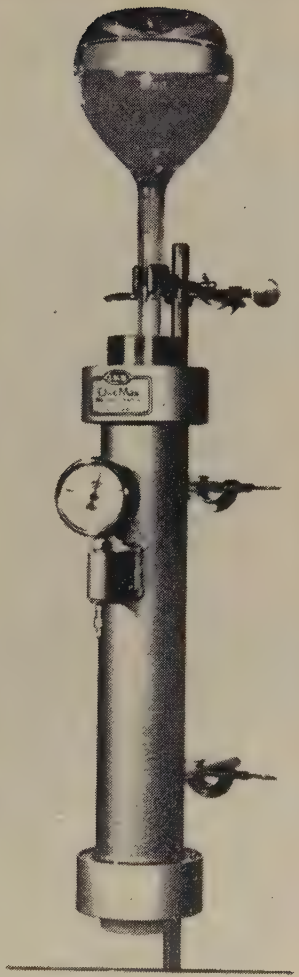

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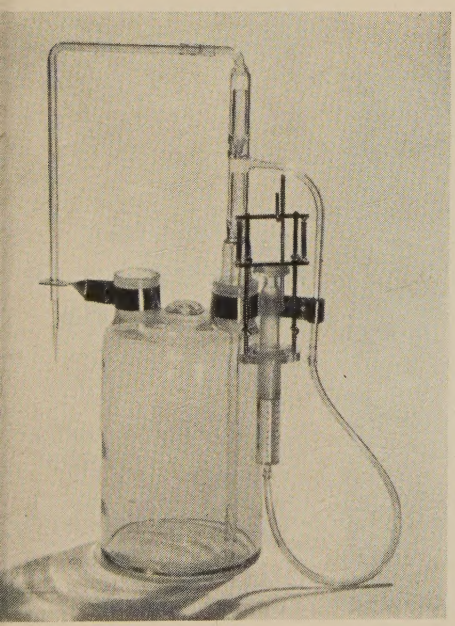

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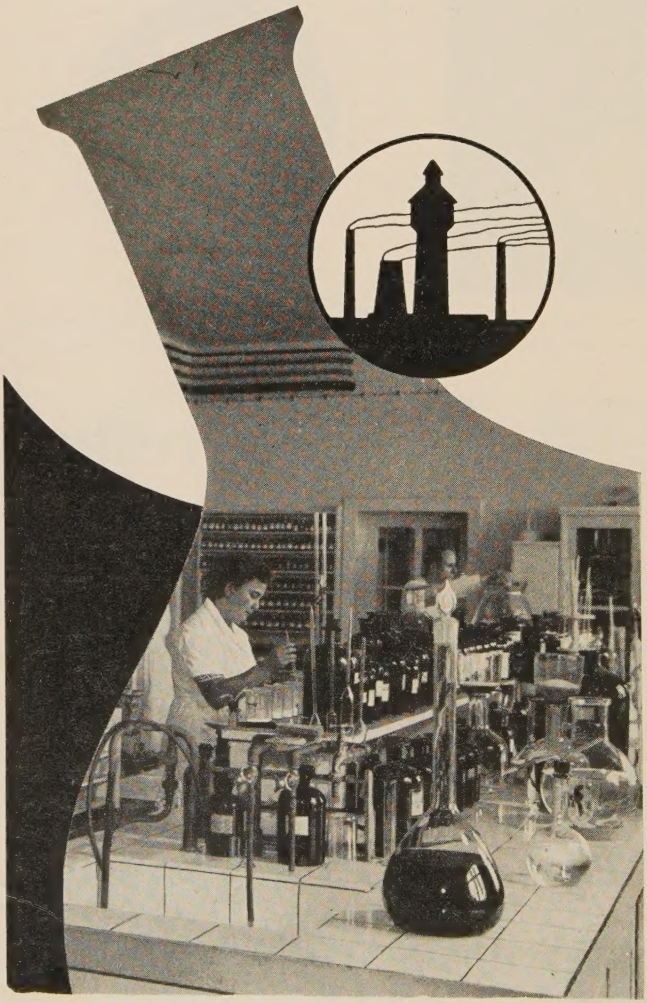

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